

**SUGAR REGULATION OF MALATE SYNTHASE  
AND ISOCITRATE LYASE GENE EXPRESSION  
IN CUCUMBER (*Cucumis sativus*)**

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### **Declaration.**

I declare that this thesis is my own composition and describes work carried out by myself. Research carried out by other parties is clearly acknowledged.

## Abstract

The glyoxylate cycle is known to take part in the net conversion of storage lipids to sugar in germinating oilseeds. Two enzymes are exclusive for this cycle, malate synthase (MS) and isocitrate lyase (ICL) and their synthesis is coordinately regulated. These enzymes are active during postgerminative growth of seeds but are repressed in mature plants. However, they appear again when plants senesce. Genes for both enzymes are regulated by carbohydrate status. The aim of this study was to examine carbohydrate regulation. Expression of *Ms* and *Icl* genes in cucumber roots was low but increased upon excision and dark-incubation during a six day period in the absence of exogenous sugar. However, when sucrose was added to the incubation medium their expression was repressed. Hairy roots obtained using *Agrobacterium rhizogenes* strain A4 showed the same pattern of expression. Transgenic hairy roots containing *Ms* and *Icl* promoters fused to the GUS reporter gene, had a low level of GUS activity. This GUS activity increased dramatically when roots were excised and incubated in the absence of sugar, indicating regulation at the transcriptional level. Histochemical staining showed that GUS activity is concentrated in root tips and lateral root primordia where demand for carbohydrate is presumably greatest. Defoliation and shading experiments were carried out to examine the expression of *Ms* and *Icl* in roots of whole plants under natural conditions. In both cases, MS and ICL mRNA increased and roots showed a decline in sugar content. Thus, induction of *Ms* and *Icl* expression takes place in roots when supply of carbohydrate from the shoot is impaired. Results are consistent with the hypothesis that gene expression in the roots is controlled by carbohydrate supply from the shoot. Identification of regulatory elements in the *Icl* promoter required for the sugar response were made possible using transgenic cucumber hairy roots. Deletions of the *Icl* gene were assayed, which located a 200 bp region necessary for the sugar response more than 1 kbp upstream of the transcriptional start. Analysis of the germination response in transgenic *Nicotiana* plants showed that sequences downstream of the sugar response region direct a low level germination response, but sequences further upstream of the sugar response region are required for a quantitative germination response. In a separate experiment, deletions of the *Ms* gene promoter previously assayed in *Nicotiana* were found to respond similarly in cucumber roots, defining a sequence of about 35 bp necessary for the sugar response but not for expression during seed germination. Regulation of *Ms* and *Icl* at the posttranscriptional level was studied using actinomycin-D, a transcription inhibitor. Detached roots which contained abundant MS and ICL transcripts due to starvation for four days, showed a decrease in mRNA amount of about 50% after 3 hour incubation in the presence of 10 µg/ml actinomycin-D. In contrast, when sucrose was added together with actinomycin-D, MS and ICL mRNA decreased to a much lower level within 3 hours. Therefore, MS and ICL half-lives appear much shorter in the presence of sugar. Similar results were obtained with detached cotyledons. Confirmation that actinomycin-D had effectively stopped transcription was obtained by showing that it prevented light-induced expression of hydroxypyruvate reductase and rubisco genes in cotyledons. Thus, *Ms* and *Icl* genes appear to be regulated by sugars at the levels of transcription and mRNA stability.

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## ABBREVIATIONS

A	absorbance
A	adenine
bp	base pair
BSA	bovine serum albumine
C	cytosine
CAMV	cauliflower mosaic virus
cDNA	complementary DNA
CoA	coenzyme A
Ci	curie
C- terminal	carboxy -terminal
DNA	deoxyribonucleic acid
ds	double stranded
EDTA	ethenediaminetetraacetic acid (disodium salt)
G	Guanine
GUS	β-glucuronidase
ICL	isocitrate lyase
IMH	isocitrate lyase-malate synthase-homology
kbp	kilobase pair
kD	kilodalton
L	litre
M&S	murashige and Skoog
MES	(2-[N-Morpholino] ethanesulphonic acid
min	minute
MOPS	3-(N-morpholino) propanesulphonic acid.
mRNA	messenger RNA
MS	malate synthase
MU	methyl umbelliferone
MUG	methyl umbelliferyl glucuronide
NAD <sup>+</sup>	Nicotinamide adenine dinucleotide (oxidised form)
nm	nanometre, 10 <sup>-9</sup> metre
NPTII	neomycin phosphotransferase, type II
N-terminal	amino terminal
poly A	polyadenylation
p.s.i.	lbs inch <sup>-2</sup>
PTS	peroxisomal targeting signal
PVP	polyvinylpyrrolidone
<i>rbcs</i>	gene encoding the small subunit of ribulose biphosphate carboxylase
RNA	ribonucleic acid
RNase	ribonuclease
r.p.m	revolution per minute
Ri	root inducing

SDS	sodium dodecyl sulphate
T-DNA	transfer DNA
TE	Tris- EDTA
u	unit
UV	ultra violet
v/v	volume:volume
w/v	weight:volume

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# **CHAPTER ONE**

## **INTRODUCTION**

# CHAPTER ONE: INTRODUCTION

## 1.1. Introduction

The growth, development and metabolism of plants have been extensively studied in order to manipulate their properties for biotechnological application. One way of understanding these activities is through expression analysis of plant genes. Therefore, elucidating the factors which regulate their expression is crucial to understanding the processes and mechanisms controlling plant development. With the advent of molecular biology techniques, plant genes of interest can be isolated and functional *cis*-elements identified, which sets the stage for the characterisation of *trans*-acting factors that may be responsible for their activities. Detailed studies of the role and regulatory pathway of *trans*-acting factors on plant development will likely open up more diverse and exciting opportunities for the controlled manipulation of plant morphology and properties.

In cucumber, the glyoxylate cycle has been used to study gene regulation. The enzyme activities of this cycle are localised in a defined cellular compartment, the peroxisome, and the cycle functions both at specific developmental stages and in response to metabolic status. Specific to this pathway are the enzymes malate synthase (MS, EC 4.1.3.2) and isocitrate lyase (ICL, EC 4.1.3.1) which have been used for studying the regulation of plant gene expression. In this introduction, biogenesis and function of various classes of peroxisome and several aspects of plant gene regulation will be discussed.

## 1.2. Peroxisomes

### 1.2.1. Characteristics and function of peroxisomes

Peroxisomes are a class of organelles in eukaryotic cells sometimes designated as microbodies, that are the subcellular location of important metabolic

reactions. In plants, they are present in essentially all cells, bounded by a single membrane and have diameters generally from 0.2 to 1.8  $\mu\text{m}$  (Huang *et al.*, 1983). All are autonomous organelles in that they lack organellar DNA, ribosomes and internal membrane systems (Douglas *et al.*, 1973; Karmiyo *et al.*, 1982). Therefore, all microbody proteins must be encoded by the nuclear genome and synthesised elsewhere in the cell. The characteristic that distinguishes peroxisomes from other organelles is that they are the site of many  $\text{H}_2\text{O}_2$ -generating oxidative reactions in cells and were given the functional name peroxisomes to reflect their role in  $\text{H}_2\text{O}_2$  metabolism (Frederick *et al.*, 1969).

Peroxisomes are unusual among eukaryotic organelles in that their size, number and enzymatic content vary depending upon the organism, cell type and metabolic need of the organism. For example, germinating fatty seeds of some plants develop prominent organelles that contain enzymes of the glyoxylate cycle, a pathway that participates in the net conversion of fatty acids to carbohydrates (Olsen *et al.*, 1995). Because of the presence of the glyoxylate pathway enzymes, these organelles are named glyoxysomes. In another example, certain protozoans, such as *Trypanosoma brucei*, sequester enzymes of glycolysis in organelles called glycosomes, a strategy designed to increase the rate of glycolysis by increasing the effective concentration of the enzymes and substrates (Clayton *et al.*, 1995).

A general characteristic of peroxisomes is their functional versatility. In higher plants, these organelles play critical roles in a variety of metabolic processes ranging from lipid mobilisation to nitrogen transport. The involvement of these organelles in a variety of metabolic functions may reflect the fact that oxidases that are often sequestered in peroxisomes participate in a number of distinct cellular processes. For example, plant (Beever, 1979) and yeast (McCammon *et al.*, 1990) peroxisomes contain  $\beta$ -oxidation enzymes as well as enzymes involved in the utilization of two-carbon compounds. Reactions involved

in ureide metabolism occur in the peroxisomes of both plants and animals (Tolbert, 1981) and peroxisomes synthesise plasmalogen, an essential component of membranes in mammalian microbodies (van den Bosch *et al.*, 1992). Based on the data gathered, it appears that the differentiated state of the cell and the environment in which they are found, determine the specific function of a given class of peroxisome.

### **1.2.2. Classes of higher plant peroxisomes**

The diversity of functions displayed by peroxisomes has allowed four classes to be identified in higher plants. Although they all share the general characteristics, each class of peroxisomes participates in a distinct metabolic function, usually at a discrete stage of the plant life cycle. Four classes are discussed here: glyoxysomes found in the fat storing cells of oil seeds, leaf peroxisomes found in the photosynthetic tissues, peroxisomes involved in ureide metabolism in the uninfected cells of root nodules of legumes and unspecialised peroxisomes.

#### **1.2.2.1. Glyoxysomes**

These specialised peroxisomes play a critical role in lipid mobilisation. Glyoxysomes are designated as such because, with the exception of cytosolic aconitase (Courtois *et al.*, 1993; De Bellis *et al.*, 1994), they possess all the glyoxylate-cycle enzymes and  $\beta$ -oxidation enzymes ( Breidenbach *et al.*, 1967; Cooper *et al.*, 1969). These enzymes catalyse the net conversion of fatty acids to succinate and are largely responsible for the ability of plants to utilise lipids as a carbon source for growth prior to the development of photosynthetic organs

(Beevers, 1980). Succinate generated by these reactions is converted to carbohydrates by gluconeogenic enzymes in the cytosol and mitochondria. The  $\beta$ -oxidation enzymes are thought to be localised extensively in peroxisomes (Gerhardt, 1986), although some reports suggest that  $\beta$ -oxidation may also occur in mitochondria (Wood *et al.*, 1984; Dueaide *et al.*, 1993).

Glyoxysomes have been characterised most extensively during the postgerminative development of oil seed plants, where they are involved in mobilising storage lipids to provide carbon for growing seedlings. Functional glyoxysomes also occur in senescent organs, presumably in response to the mobilisation of membrane lipids (Gut and Matile, 1988; De Bellis *et al.*, 1991). Additionally, the activities of two enzymes associated exclusively with glyoxysomes, isocitrate lyase (ICL, EC 4.1.3.1) and malate synthase (MS, EC 4.1.3.2) have been detected in developing seeds (Comai *et al.*, 1989; Turley *et al.*, 1990; Ettinger *et al.*, 1990) and pollen (Zhang *et al.*, 1994). However, it is unclear whether the other glyoxylate cycle enzymes are also present at these stages of the life cycle.

#### **1.2.2.2. Leaf-type peroxisomes**

Leaf peroxisomes are present in photosynthetically active tissue such as green cotyledons and leaves (Frederick *et al.*, 1969) reflecting the cooperative roles of these organelles in photorespiration. Photorespiration is the oxygen- and light-dependent evolution of carbon dioxide that is associated with the reactions of photosynthesis, particularly in  $C_3$  plants (Orgen *et al.*, 1984). The metabolic reactions involved in photorespiration are speculated either to dissipate excess photosynthetic capacity that cannot be used for carbon fixation or to recover carbon redirected from the photosynthetic carbon reduction cycle as a result of

ribulose biphosphate oxygenase activity. Ribulose biphosphate carboxylase (Rubisco) normally catalyses the carboxylation of ribulose 1,5-bisphosphate (RuBP) which leads to the formation of two molecules of glycerate 3-phosphate and the continuation of the Calvin cycle of photosynthesis. During photorespiration however, Rubisco oxygenates RuBP to form glycerate phosphate and glycollate 2-phosphate. Glycolate 2-phosphate cannot be utilised in the Calvin cycle and would represent a functional loss of assimilated carbon if not further metabolised. However, some of the carbon may be recycled via the photosynthetic carbon oxidation cycle (PCO or glycolate pathway). Leaf-type peroxisomes contain the photorespiratory enzymes, glycolate oxidase, serine-glyoxylate aminotransferase and hydroxypyruvate reductase which convert glycolate to glycine, and serine to glycerate. Those enzymes rise in amount from undetectable levels to major peroxisomal components as the cotyledons green and gain photosynthetic capacity. Accumulation of these enzymes is regulated primarily at the transcriptional level and is light dependent (Hondred *et al.*, 1987; Greenler *et al.*, 1990).

#### **1.2.2.3. Root nodule peroxisomes**

Peroxisomes present in the root nodules of many nitrogen-fixing plants play a role in reactions involved in nitrogen transport (Reviewed in Schubert, 1986). Many tropical legumes such as soybean and cowpea, transport nitrogen in the form of the ureides, allantoin and allantoic acid, which are derived from purine catabolism. Reactions involved in ureide biosynthesis are predicted to occur in several cellular compartments of two nodule cell types (cells infected with nitrogen-fixing rhizobia and uninfected cells). In infected cells, fixed ammonia is assimilated in the cytosol and the purine-based xanthine is synthesised in the plastids. The subsequent conversion of xanthine to allantoin and allantoic acid is

thought to occur in uninfected cells (Huang *et al.*, 1983). One of the final steps in this pathway, the conversion of urate to allantoin is catalysed by urate oxidase and occurs in peroxisomes (Nguyen *et al.*, 1985).

#### **1.2.2.4. Unspecialised peroxisomes**

Unspecialised peroxisomes are present in plant tissues that are not active photosynthetically and that lack storage lipids (reviewed in Huang *et al.*, 1983). These organelles contain catalase and in most cases, H<sub>2</sub>O<sub>2</sub>-producing oxidases. Unspecialised peroxisomes are smaller than glyoxysomes or leaf-type peroxisomes and occur less frequently than those other types of peroxisome. Their buoyant density is lower than that of either glyoxysomes or leaf-type peroxisomes (Huang *et al.*, 1971). However, the specific role of these organelles in cellular metabolism is not clearly understood. One postulation would be that these peroxisomes dissipate excess energy in the cells or protect cells from oxygen toxicity at high oxygen concentration (reviewed in Huang *et al.*, 1983).

#### **1.2.3. Origin of specialised classes of peroxisomes**

Each specialised peroxisome has its specialised function catalysed by a characteristic set of enzymes (Olsen and Harada, 1991), suggesting that the metabolic role of a specific class of peroxisome is determined by the mechanisms that regulate enzyme accumulation within the organelle. Although accumulation of individual peroxisomal enzymes is regulated at both transcriptional and postranstriptional levels, (Comai *et al.*, 1989; Ettinger and Harada, 1990; Graham *et al.*, 1990), the protein import mechanism does not appear to play a role in determining organelle function. In other words, specialised peroxisomes do not



selectively import specific classes of peroxisomal protein. In particular, glyoxysomal enzymes, which are not normally present in leaves and roots, are imported into leaf-type and root peroxisomes when expressed in transgenic plants (Olsen *et al.*, 1993; Onyeocha *et al.*, 1993). Furthermore, the peroxisomal protein firefly luciferase can be imported into peroxisomes from yeast, insects, mammals and plants (Keller *et al.*, 1991). Together, these data clearly show that classes of peroxisome are competent to import all peroxisomal proteins, which suggests the existence of a common import pathway.

Further strong evidence in support of the common mechanism of peroxisome formation is that peroxisomes of one functional class can be converted into peroxisomes with a different metabolic role. The best characterised example is the conversion of glyoxysome to leaf peroxisome, which occurs as seedlings undergo the transition from heterotrophic to autotrophic growth. In cotyledons undergoing this transition, individual peroxisomes containing enzymes characteristic of both glyoxysomes and leaf-type peroxisomes have been identified (Nishimura *et al.*, 1986; Sauther, 1986; Titus and Becker, 1985). These data suggest that the conversion of peroxisomal function results from a replacement of glyoxysome-specific enzymes with those characteristic of leaf-type peroxisomes. Additional support for this postulation has been obtained from immunocytochemical analysis of senescing cotyledons. In this experiment, leaf-type peroxisomal enzymes and glyoxysomal enzymes are detected during cotyledon senescence when leaf-type peroxisomes are converted to glyoxysomes (Nishimura *et al.*, 1993). These findings indicate that a common mechanism of assembly may be used in formation of peroxisomes with different functions.

#### 1.2.4. Model of peroxisome formation

Two models have been proposed to explain how peroxisomes are formed. Originally based on ultrastructural data, peroxisomes were thought to derive by budding from the endoplasmic reticulum (ER), but now it is generally accepted that new peroxisomes originate from the growth and division of preexisting peroxisomes. Organellar growth is accomplished through the addition of lipids and the posttranslational import of protein (Lazarow and Fujiki, 1985; de Hoop and Ab, 1992). All peroxisomal matrix and membrane proteins analysed to date are synthesized in the cytosol and transported posttranslationally directly into the organelle (Fujiki *et al.*, 1984; Lazarow *et al.*, 1991; Osumi and Fujiki, 1990).

An important breakthrough in research on peroxisome biogenesis has been the application of yeast genetics. This is particularly due to the ability to control the proliferation and enzyme composition of their organelles via growth substrate manipulation (Veen Huis and Harder, 1991). In *Hansenula polymorpha* or *Pichia pastoris*, growth on methanol induces the synthesis of large amounts of three peroxisomal enzymes: alcohol oxidase, catalase and dihydroxypyruvate synthase. When shifted to methanol, the peroxisomes which are normally small and few, undergo massive proliferation (Gregg *et al.*, 1990; Liu *et al.*, 1992). In other yeasts including *Saccharomyces cerevisiae*, (Erdman *et al.*, 1989) *P. pastoris*, (Gould *et al.*, 1992) and *Yarrowia lipolytica* (Nuttley *et al.*, 1993) growth on fatty acids (such as oleic acid) induces the appearance of numerous large peroxisomes containing  $\beta$ -oxidation pathway enzymes.

Peroxisomes do not appear to form *de novo* in the cell (Lazarow and Fujiki, 1985; Subramani, 1993). This supports the peroxisome fission hypothesis that new peroxisomes cannot arise unless pre-existing peroxisomes are present to produce them. In *S. cerevisiae* cells grown on glucose, at least one small peroxisome exists

which grows and proliferates when glucose is removed from the growth medium (Aitchison *et al.*, 1992). However, transformation of yeast and mammalian *pex* (peroxisome-deficient) mutants with the appropriate wild-type genes readily restores normal peroxisomes. It has been suggested that the *pex* mutants, although without peroxisomes, retain remnants of peroxisomes that serve as the starting material to initiate the peroxisome regeneration process (Purdue and Lazarow, 1995). A recent study suggests, however, that peroxisomal remnants do not participate in the regeneration of peroxisomes (Waterham *et al.*, 1993). The study utilised a temperature-sensitive *pex* (*pex<sup>ts</sup>*) mutant of *H. polymorpha* that lack peroxisomes at nonpermissive temperature but does contain peroxisomal remnants in the form of phospholipids aggregates. Upon shifting the *pex<sup>ts</sup>* mutant to the permissive temperature, normal peroxisomes rapidly reappeared. A heterologous membrane protein used to mark the remnants was not transferred to newly formed organelles, suggesting that the remnants did not contribute to peroxisome formation. This suggests that another peroxisome generation mechanism, that is independent of preexisting peroxisomes, may exist.

Recently, HpPex10P a peroxisomal integral-membrane protein from *H. polymorpha* (Tan *et al.*, 1995) and ScPex11P, a peroxisomal membrane associated protein from *S.cerevisiae* (Erdman *et al.*, 1995; Marshall *et al.*, 1995) were described to be the first two components for peroxisome proliferation. This was suggested by the observation that their overexpression leads to an overproliferation of peroxisomes. Furthermore, oleate-induced cells of a *S.cerevisiae* *pex11*-deleted strain contained a single large peroxisome, the phenotype of a proliferation-defective mutant. It appears that for *PEX10* and specially for *PEX11*, there is a direct correlation between the levels of their products and the number of peroxisomes in each cell, suggesting that Pex10P and Pex11P are somehow involved.

### 1.2.5. Mechanism of protein import

Like many mitochondrial and chloroplast proteins, peroxisomal proteins are synthesised on cytoplasmic ribosomes and posttranslationally imported into the organelles by a process that requires ATP and is aided by *hsp70*-class cytoplasmic chaperones (Subramani, 1993; Walton *et al.*, 1994). Nevertheless, unlike mitochondrial and chloroplastic protein import, most peroxisomal proteins do not undergo proteolytic processing. Therefore, the amino acid sequences within these proteins that target them to the peroxisome (the peroxisomal targeting signals ) must reside within the mature polypeptide. Peroxisomal proteins such as malate dehydrogenase (MDH) (Gielt, 1990), pumpkin catalase (Yamaguchi *et al.*, 1984), yeast carnitine acetyltransferase (Ueda *et al.*, 1984) and thiolase from cucumber (Preisig-Muller and Kindl, 1993) are synthesised with an amino-terminal peptide that is proteolytically cleaved after import. This process has not been demonstrated to be tightly coupled to translocation (Miura *et al.*, 1984). Other peroxisomal proteins apparently have neither a carboxyl-terminal nor amino terminal targeting signal. Thus, distinct signals have been localised to several different regions of peroxisomal proteins.

#### 1.2.5.1. Carboxyl-terminal signal

The first and most common of these peroxisomal targeting signals (PTS) is PTS1, carboxy-terminal tripeptide of the sequence Ser-Lys-Leu (SKL), which was first identified on firefly luciferase (Gould *et al.*, 1989). An experiment using double immunofluorescence microscopy with anti-catalase antibodies, as a peroxisomal marker, has shown that when attached to the carboxyl terminus of non-peroxisomal proteins, PTS1 is sufficient to direct these fusion proteins to the organelles (Gould *et al.*, 1989). The SKL signal was also identified functionally in

rat acyl-CoA oxidase, using import experiments with isolated peroxisomes (Miyazawa *et al.*, 1989). Substitution of each of the amino acids of the tripeptide signal has demonstrated that certain conservative changes can be tolerated without a loss of targeting activity (Gould *et al.*, 1989).

A number of plant peroxisomal proteins have a related carboxyl-terminal tripeptide. For example, the glyoxysomal protein isocitrate lyase, includes the carboxyl-terminal amino acids serine-arginine-methionine, which was shown to be both necessary and sufficient for import into peroxisomes of transgenic plants (Olsen *et al.*, 1993). The PTS1 import machinery has been conserved through evolution, as demonstrated by the observation that luciferase, as well as other proteins with the PTS1 motif, are imported into peroxisomes in mammals, insects, plants, *T. brucei* (glycosomes) and yeast. Furthermore, antibodies raised against SKL recognise peroxisomal proteins in each of these organisms (Gould *et al.*, 1990; Keller *et al.*, 1991). Unlike signal sequences on proteins destined for most other organelles, PTS1 is not cleaved after import.

Several peroxisomal proteins, including peroxisomal catalase, do not have a carboxyl-terminal SKL motif, but possess an acceptable tripeptide upstream of the carboxyl terminus (Gonzales *et al.*, 1991). Nevertheless, no definitive evidence indicates that a SKL-like tripeptide can function as a targeting signal at an internal position in a polypeptide. The targeting signal could be inactivated by the addition of as few as one amino acid to the carboxyl terminus of the firefly luciferase SKL (Gould. *et al.*, 1989). In the case of *H. polymorpha*, a SKL-like tripeptide near the carboxyl terminus of the peroxisomal protein amine oxidase has been shown not to be involved in targeting (Bruinenberg *et al.*, 1989; Roggenkamp, 1992). These data show the possibility that internal SKL-like tripeptide can have a role in protein targeting but that the internal signals may function only within a specific sequence or only in a particular protein conformation. Targeting of luciferase to

peroxisomes was partially affected by a variety of mutations that did not eliminate the carboxyl-terminal targeting signal (Gould *et al.*, 1987).

#### 1.2.5.2. Amino-terminal signal

The second protein targeting sequence, PTS2, is an amino-terminal motif with consensus sequence Arg-Leu-X<sub>5</sub>-His/Gln-leu (RLX<sub>5</sub>H/QL), which was first identified on rat thiolase (Swinkels *et al.*, 1991). PTS2 is less common than PTS1 but also appears to be conserved since it is found on peroxisomal proteins from mammals (Subramani, 1993), watermelon and cucumber glyoxysomal malate dehydrogenase (gMDH) (Gielt *et al.*, 1994; Kim and Smith, 1994b), trypanosome aldolase (Blatter *et al.*, 1994), and *Hansenula polymorpha* amine oxidase (Faber *et al.*, 1995). Interestingly, the amino terminus containing PTS2 is cleaved after import in some instances (rat thiolase, watermelon malate dehydrogenase) but not in others (yeast thiolase and amine oxidase).

The PTS2 peptide of thiolase from cucumber, *Arabidopsis thaliana*, rat and human are very similar to each other. They also display some similarity to the peptide from watermelon malate dehydrogenase and to the first 11 amino acids of peroxisomal amine oxidase from *H. polymorpha*, which suggests that they may share similar targeting roles (Bruinenberg *et al.*, 1989; de Hoop and Ab, 1992). The consensus sequence of Arg-Leu-X<sub>5</sub>-His/Gln-Leu (RLX<sub>5</sub>H/QL) was suggested by Glover *et al.* (1994), but has not been well defined. This consensus sequence located in the amino terminal region of aldolase from *Trypanosoma brucei* is found to be sufficient to direct a cytosolic protein into microbodies (Glover *et al.*, 1994). Furthermore, arginine and both leucines of the consensus sequences were shown to be critical for targeting thiolase to *Saccharomyces cerevisiae* peroxisomes although the amino terminus is not cleaved upon import.

### 1.2.5.3. Internal signal

Since many peroxisomal enzymes have neither a PTS1 nor PTS 2 motif, it is likely that other PTS sequences remain to be discovered. Support for the existence of additional PTS sequences comes from targeting studies with acyl-coA oxidase from *C. tropicalis* and catalase A from *S.cerevisiae*, which indicate that these enzymes are imported via internally located PTS sequences that have no obvious similarity to PTS1 or PTS2 (Kragker *et al.*, 1993; Small *et al.*, 1988). Acyl-coA oxidase from *C. tropicalis* contains two separate domains, one amino-terminal but not cleaved, and a second located in the central portion of the polypeptide. Each domain was sufficient to target a passenger protein to peroxisomes using an *in vitro* import assay and was itself imported into peroxisomes in transgenic *Candida maltosa*. Neither of these regions includes a recognisable SKL-like peptide (Small *et al.*, 1988). *S.cerevisiae* catalase A contains two domains that are sufficient to direct a reporter protein to peroxisomes, a large amino-terminal domain and a small carboxy-terminal region (Kragker *et al.*, 1993). The *Hansenula polymorpha* *PER1* gene encodes a protein, PerP1, which possesses both a PTS1 and a PTS2, either of which is capable of targeting the protein to peroxisomes. Thus, it is suggested that in some cases, multiple signals are present in a single protein, each of which is capable of targeting the protein to peroxisomes. This functional redundancy may increase efficiency (Miyazawa *et al.*, 1989).

Experiments with glyoxysomal ICL provide another potential example of internal signals that function in targeting. The last 37 amino acids of *Brassica napus* ICL, including a putative PTS1 at the extreme C-terminus, are necessary for import of this protein into *Arabidopsis* leaf peroxisomes and the last 5 amino acids are sufficient to direct chloramphenicol acetyl transferase to peroxisomes (Olsen *et al.*, 1993). However, the almost identical carboxy-terminal sequence is not

required for import of *Ricinus communis* ICL into isolated sunflower cotyledon glyoxysomes (Behari and Baker, 1993).

#### 1.2.5.4. Protein targeting receptors

The presence of PTSs on peroxisomal proteins implies the existence of receptors that recognise and bind to these sequences. Potential PTS1 and PTS2 receptor proteins have been identified (Raccubinski and Subramani, 1995). Ppex5P of *P. pastoris* was first demonstrated as the PTS1 receptor. Mutation of the *PEX5* gene in *P. pastoris* and other organisms would lead to failure of PTS1 translocation but does not affect the import of the PTS2 imported enzyme thiolase. PpPex5P is predicted as a 68-kD polypeptide with seven copies of a motif called the snap helix or tetratricopeptide repeat which binds peptides ending with SKL with high affinity and specificity *in vitro* (Terlecky *et al.*, 1995). In human peroxisomes, Pex5P has the same characteristic as in *P. pastoris* and was first identified by the similarity of their predicted amino acid sequences (Fransent *et al.*, 1995). Mutation of this gene is responsible for the fatal peroxisome disorder, called Zellweger syndrome (Walton *et al.*, 1992).

A PTS2 receptor, Pex7P, has been identified in *S. cerevisiae*, based on the phenotype of the mutants resulting from its mutated *PEX7* gene. *Pex7* mutants have morphologically normal peroxisomes that import PTS1 enzymes but not the PTS2 thiolase, the opposite phenotype of the PTS1 receptor mutants (Marzioch *et al.*, 1994). Pex7P is predicted to be a 42-kD protein with six copies of a motif of approximately 40 residues, called the WD repeat, named for the conserved presence of a Trp-Asp (WD) pair at the carboxy terminus of each copy of the motif (Rehling *et al.*, 1996). In humans, deficiency in PTS2-mediated peroxisomal



import is responsible for the human peroxisomal disorder Rhizomelic chondrodysplasia punctata (Slawecki *et al.*, 1995).

#### 1.2.6. General characteristics of the protein import machinery

Generally, little is known about the mechanism of import. However, translocation of glycolate oxidase (Horng *et al.*, 1995) and acyl-CoA oxidase into peroxisomes (Imanaka *et al.*, 1987), castor bean ICL into sunflower glyoxysomes (Behari and Baker, 1993) and firefly luciferase into mammalian peroxisomes has been shown to require ATP hydrolysis. In some systems, N-ethylmaleimide-sensitive factors are also believed to be involved (Wendland and Subramani *et al.*, 1993). In addition, ATPase activities, all with different inhibitor sensitivities have been detected in peroxisome of yeast (Whitney and Bellio, 1991) and rat liver (del Valle *et al.*, 1988). The PAS 1 protein from *S.cerevisiae* (Erdman *et al.*, 1991) and PAY 4 protein of *Yarrowia lipolytica* (Nuttley *et al.*, 1994) have been shown to possess a highly conserved consensus sequence for ATP binding and appear to belong to a novel family of putative ATPase.

PTS1 and PTS2 are strongly believed to have the same import machinery. Upon binding to a PTS specific receptor, these matrix proteins are channelled through a common translocation apparatus. It was generally assumed that proteins enter the peroxisomal matrix by passing through a translocation channel in a monomer conformation but then change into mature oligomeric structure after being imported into peroxisomes (McNew and Goodman, 1994). Import of thiolase in *S. cerevisiae*, a homodimer, is prevented by removal of its PTS. However if this PTS-less thiolase is co-expressed along with full-length thiolase, both are efficiently imported (Glover *et al.*, 1994). Analagous result have been obtained with a PTS1-containing protein as well (Hauster *et al.*, 1996). These results suggest that polypeptides can be imported in oligomeric form.

## 1.3. Glyoxylate cycle

### 1.3.1. The Glyoxylate cycle activity

The glyoxylate cycle, a modified form of the tricarboxylic acid cycle, takes place in most plants and microorganisms. The primary role of the glyoxylate cycle is to enable plants and microorganisms to utilize fatty acids or acetate, in the form of acetyl-CoA as sole carbon source, particularly for the biosynthesis of carbohydrate from fatty acid. This process was first identified in *Pseudomonas* which used acetate as sole carbon source to grow (Kornberg and Madsen, 1957). In higher plants, the cycle is responsible to meet the need for plant development by converting two acetyl-CoA molecules derived from  $\beta$ -oxidation of fatty acid into succinate, and this was first observed in endosperm tissue of germinating castor bean (Kornberg and Beevers, 1957). During postgerminative growth, seed lipid reserves undergo rapid mobilisation through lipase and  $\beta$ -oxidation activities to produce acetyl-CoA. The glyoxylate cycle then mediates the conversion of acetyl-CoA to succinate, and this is subsequently converted to carbohydrate.

Both the tricarboxylic acid cycle and glyoxylate cycle use the same reactions in the initial steps. Oxaloacetate (OAA) and acetyl-CoA undergo condensation to form citrate through the action of citrate synthase. Citrate is then converted to isocitrate by the enzyme aconitase. However, the break down of isocitrate through subsequent decarboxylation as in the tricarboxylic acid cycle is by-passed by the action of two enzymes exclusive to the glyoxylate cycle, ICL and MS. Isocitrate is first cleaved by ICL to form succinate and glyoxylate. MS then catalyses the condensation of glyoxylate with a second molecule of acetyl-CoA to form malate. Other than MS and ICL, citrate synthase, malate dehydrogenase and aconitase are central to the glyoxylate cycle (Cooper and Beevers, 1969).

Investigation of glyoxysomes from a variety of plants tissues have established that they all have a similar enzyme complement and that they play a role in the conversion of fatty acids to sugar. Thus, the sequence of reactions whereby long chain fatty acids are converted to succinate by way of  $\beta$ -oxidation and the glyoxylate cycle are found in the glyoxysome (Breidenbach and Beevers, 1967). However, evidence suggests that aconitase, is absent from glyoxysomes (Courtois-Verniquet and Douce, 1993; De Bellis *et al.*, 1994).

### 1.3.2. Synthesis of MS and ICL during plant development

The enzymes ICL and MS are unique to the glyoxylate cycle and have been studied extensively as marker enzymes for this cycle during plant development. Their activities have been detected in higher plants during embryogenesis and seed germination of many plants (Allen *et al.*, 1988; Comai *et al.*, 1989; Turley and Trelease, 1990; Weir *et al.*, 1980; Zhang *et al.*, 1993), senescence (Graham *et al.*, 1992; Pistelli *et al.*, 1991; De Bellis *et al.*, 1991) and pollen formation (Zhang *et al.*, 1994).

In cucumber, MS is composed of eight identical subunits (Koller and Kindl, 1977) and appears to be encoded by a single copy gene (Graham *et al.*, 1989). In *B. napus*, MS is encoded by several genes which fall into four different classes but only a single class appears to be expressed during germination (Comai *et al.*, 1992).

Two subunits of ICL, 63 and 61.5 kDa, were identified in cucumber (Weir *et al.*, 1980; Reizman *et al.*, 1980). Similar to MS, cucumber ICL appears to have a single gene (Reynolds and Smith, 1995a). In *B. napus*, there are six *Icl* genes which can be classified into two subfamilies according to 5' and 3' untranslated

sequences. Both classes are expressed during seed development (Zhang *et al.*, 1993).

The regulation of both of these enzymes is thought to be primarily at the level of transcription (Comai *et al.*, 1989; Allen *et al.*, 1988) and the levels of enzyme activity reflect changes in levels of mRNA (Smith and Leaver, 1986). Approximately 1 kb of the 5' region of the cucumber *Ms* gene has been shown to direct expression of a reporter gene in transgenic plants in the same temporal and spatial pattern as the gene is regulated in cucumber (Graham *et al.*, 1990). An *Icl* promoter fragment of length 2.9 kb was linked to the GUS gene and transferred into *Nicotiana plumbaginifolia*. Analysis of GUS activity indicated that the *Icl* promoter fragment faithfully directed the temporal regulation of *Icl* gene expression during postgerminative growth. However, regulation of gene expression may also occur at the posttranscriptional level as the ratio of MS protein to mRNA is 14-fold higher than that for ICL, a difference which does not seem to be accounted for by differences in the translational efficiencies (Ettinger and Harada, 1990). In certain microorganisms, phosphorylation and dephosphorylation seems to affect regulation of ICL activity. For example, in *Escherichia coli*, the enzyme is active in the phosphorylated form, but in *Saccharomyces cerevisiae*, it is the dephosphorylated form which is active (Hoyt and Reeves, 1988; Robertson and Reeves, 1989; Lopez-Boado *et al.*, 1988). In plants, ICL of castor bean and cucumber is phosphorylated by a glyoxysomal protein kinase but its effect on ICL activity remains to be elucidated (Finnessy *et al.*, 1994).

### 1.3.2.1 *Ms* and *Icl* gene expression during postgerminative growth

During post germinative growth, many seeds depend solely on storage lipid as a carbon source until photosynthetic organs are produced. At this stage, the glyoxylate cycle is active and plays an important role in the conversion of triacylglycerol into carbohydrate. In plants it was first demonstrated to operate at this stage in castor bean endosperm (Kornberg and Beevers, 1957). Other studies on the regulation of synthesis of MS and ICL enzymes in the glyoxylate cycle during germination have shown that these enzymes are most active during post-germinative growth when storage triacylglycerols are converted to acetyl-CoA through the action of lipase and  $\beta$ -oxidation in oilseed rape (Comai *et al.*, 1989), cucumber (Becker *et al.*, 1978; Reizman *et al.*, 1980; Weir *et al.*, 1980), sunflower (Allen *et al.*, 1988) and cotton (Turley and Trelease, 1990).

Upon germination of fat-storing seeds, the activity of MS and ICL increases from a very low amount to a peak level after a few days of growth (Carpenter and Beevers 1959). During early post-germinative growth of cucumber, ICL and MS enzyme activities are found to rise in parallel to a peak at four days after seed imbibition, then fall to an undetectable level by day seven (Becker *et al.*, 1978). Monospecific antibodies have been used to detect these two proteins among the products of *in vitro* translation programmed with cotyledonary RNA from cucumber (Lamb *et al.*, 1978; Reizman *et al.*, 1980). Immunoprecipitation of ICL and MS from *in vitro* translation products of cotyledonary RNA isolated during the first seven days of post-germinative growth, indicated that the mRNA levels for each of these enzymes rise and fall in a similar pattern to enzyme activities (Weir *et al.*, 1980).

Northern hybridisation experiments using a cucumber *Ms* cDNA probe together with Western blot and enzyme activity assays have shown that regulation

of MS synthesis during post-germinative growth is brought about primarily through changes in the levels of transcripts rather than through control of translation (Smith and Leaver, 1986). A fragment containing a *Ms* putative promoter sequence was fused to the GUS reporter gene and transferred into *Nicotiana plumbaginifolia*. Analysis of GUS activity showed a peak similar to that observed for MS activity in cotyledons during germination. This showed that proximal upstream *cis*-acting sequences of the cucumber *Ms* gene contain sufficient information to direct faithful expression of the gene, and further support the proposal that regulation of MS mRNA levels in cotyledons during germination is primarily a result of transcriptional control (Graham *et al.*, 1990).

ICL mRNA of sunflower disappeared from illuminated seedlings more quickly than dark grown seedlings, thus suggesting *Icl* expression in sunflower cotyledons is developmentally regulated and modulated in seedlings by exposure to light. The sunflower ICL mRNA accumulated 24 h before ICL protein was first detectable and later declined (Allen *et al.*, 1988). Steady state levels of MS and ICL mRNA in dark-grown seedlings exhibited peaks approximately 24 h prior to the peak of their corresponding activities and protein (Turley and Trelease, 1990).

A similar relationship was also reported in light-grown cucumber seedlings (Weir *et al.*, 1980). Smith and Leaver (1986) found that changes in the amounts of *Ms* transcript and polypeptides were closely coordinated in light and dark-grown seedlings. In *B.napus*, nuclear run-off experiments suggested that the increase in transcriptional activity of the *Ms* and *Icl* genes was primarily responsible for the increase in their mRNA levels. MS and ICL activities in seedlings were found to be approximately the same but the quantitative ratio of the MS protein to its mRNA was significantly higher than that for ICL (Ettinger and Harada, 1990), thus suggesting translational or post-translational processes may affect the regulation of MS and ICL activities.

#### 1.3.2.2. MS and ICL synthesis in seed maturation

The activity of both MS and ICL was detected in maturing sunflower seeds (Fusseder and Theimer, 1984), although the ICL activity could only be found in the supernatant of sucrose density gradients, not in crude homogenates. Miernky *et al.* (1979) examined numerous oil seeds and found MS activity, but not ICL in dry seed extracts. In maturing sunflower seeds, ICL mRNA was detected using dot blots (MS was not examined), but ICL protein was not detected at the same stage of seed development on western blots using antiserum (Allen *et al.*, 1988). The activities of MS and ICL occur together in developing cotton seeds but the pattern of these two enzyme activities is not clearly coordinated as they are during post-germinative growth (Turley and Trelease, 1990). However, MS and ICL mRNAs appear to be coordinately regulated during embryogeny in oilseed rape (Comai *et al.*, 1989).

During embryogenesis and post-germinative growth, more than one class of *Icl* and *Ms* gene is expressed in *B. napus* but the accumulation of ICL and MS at these stages is not due to alternate expression of different *Icl* and *Ms* genes (Zhang *et al.*, 1993; Comai *et al.*, 1992). Deletion analysis of promoters of *B. napus* fused to the GUS reporter gene have shown that different DNA sequences activate *Icl* gene expression during late embryogenesis and postgerminative growth (Zhang *et al.*, 1996).

#### 1.3.2.3. *Ms* and *Icl* gene expression in senescence

Senescence is a complex, highly regulated, developmental phase in the life of a leaf that results in loss of cellular structure and function (Thimann, 1980; Thompson *et al.*, 1987). During this period, massive and co-ordinated degradation

of macromolecules and the subsequent mobilisation of components such as nitrogen, carbon and minerals will take place. This process is a highly regulated, ordered series of events involving cessation of photosynthesis, disintegration of chloroplasts, breakdown of leaf protein, loss of chlorophyll and removal of amino acids (Paliyath and Droillard, 1992). Leaf senescence does not occur by a passive decay mechanism, but rather by an actively regulated process that involves co-ordinated expression of specific genes. During senescence, the levels of total RNA decrease and the expression of many genes is switched off (Bate *et al.*, 1991; Hensel *et al.*, 1993; Lohman *et al.*, 1994). However, it has been postulated that the senescence process may depend on *de novo* transcription of nuclear genes. New transcripts were originally detected as a change of products observed in *in vitro* translation experiments using mRNA from senescing leaves when compared to green leaves (Malik, 1987; Thomas *et al.*, 1992).

The level of key enzymes of the glyoxylate cycle, MS and ICL have been shown to increase in senescing leaves of barley after excision and incubation in the dark. The activity increased up to day six of treatment and thereafter declined. Prior to detachment, the level of activity could hardly be detected (Gut and Matile, 1988). These enzyme activities were coupled with a decline in monogalactosyl diacylglycerol, a thylakoid lipid, suggesting that the glyoxylate cycle may be involved in the degradation of structural lipids. Furthermore, De Bellis *et al.* (1990) demonstrated similar peaks of MS and ICL activity in peroxisomes of dark-treated rice and leaf-beet leaves and pumpkin cotyledons. Data from Pistelli *et al.* (1991) confirm the presence of MS and ICL in wheat and rice leaves during natural senescence and after dark treatment of whole plants. Moreover, using fractionated cellular organelles, MS and ICL activities were detected in peroxisomal fractions from petals only of the senescing stage (De Bellis *et al.*, 1991), thus confirming the finding of Graham *et al.* (1992) on the presence of MS mRNA in naturally senescing cotyledons, leaves and petals from cucumber. MS mRNA has also been



found to appear in senescing leaves, cotyledons and roots by excision and dark treatment (Graham *et al.*, 1992; McLaughlin and Smith, 1994). Accumulation of MS mRNA in artificial senescence has been investigated using leaves of transgenic *Nicotiana plumbaginifolia* containing the cucumber *Ms* promoter fused to the GUS reporter gene (Graham *et al.*, 1990). GUS activity was shown to appear as early as day six thus suggesting that *Ms* gene transcription is activated rather than a decrease in MS mRNA turnover.

It appears that cellular lipids, such as the galactolipids, which are the main component of the thylakoid membranes, or the endomembrane lipids, may be metabolised via the glyoxylate cycle during senescence (Vicentini and Matile, 1993; McLaughlin and Smith 1995). Sucrose can be synthesised from the four carbon products of the glyoxylate cycle via the process of gluconeogenesis, which converts TCA cycle components to sucrose via a reversal of the glycolytic pathway. This sucrose can be used for respiration or export from the senescing leaf. The co-ordinate expression of the gene encoding phosphoenolpyruvate carboxykinase (PEPCK), a key enzyme involved in the conversion of lipids to sugars, with the genes for MS and ICL during the natural senescence of cucumber cotyledons, supports the suggestion that the expression of these genes is related to gluconeogenesis (Kim and Smith, 1994a). PEPCK catalyses the conversion of oxaloacetate to PEP, an essential step for gluconeogenesis to occur. Similarly, increased expression of peroxisomal NAD malate dehydrogenase, one of the enzymes involved in the glyoxylate cycle, has been detected in senescencing cucumber cotyledons (Kim and Smith, 1994b).

## 1.4. Metabolite-regulated gene expression

### 1.4.1. Carbohydrate depletion and sugar-responsive genes

Plant and microbial gene responses to carbohydrate availability have important similarities and differences as well. In both plants and microbes, addition of sugar or acetate leads to uptake of the preferred substrate requiring the least metabolic cost, and promotes heterotrophic growth over photosynthesis. In higher plants, carbohydrate-regulated genes provide a means for optimising investment of carbon, nitrogen and phosphorus among different plant parts and processes (Madore and Lucas, 1995).

In carbon-exporting or other autotrophic cells, photosynthetic genes are typically up-regulated by sugar starvation. Among others, these include genes of the CO<sub>2</sub> fixation enzymes of both C3 and C4 plants (Sheen, 1990; Sheen, 1994). Both nuclear and plastid genes are also affected, though the latter may respond more slowly to altered carbohydrate levels (Criqui *et al.*, 1992; Shih and Goodman, 1988). Photosynthetic genes such as *Rubisco* are repressed most by acetate and often more strongly by hexose than sucrose (Sheen, 1990). Genes for remobilisation of sugar and other small molecules from polymers and vacuoles are also induced by carbohydrate starvation. In photosynthetic leaves, those genes associated with starch breakdown can be induced by carbohydrate starvation and repressed by glucose (Krapp and Stitt, 1994). A similar response is observed with the  $\alpha$ -amylase gene in rice which catalyses the hydrolysis of 1,4 endoglycosidic bonds of amylose and amylopectin to convert starch into maltose and glucose. Carbon metabolites suppress the expression of this gene in germinating seeds and suspension-cultured cells of rice but it is derepressed by starvation conditions (Yu *et al.*, 1991; Thomas and Rodriguez, 1994). In higher plants, carbon metabolite

regulation of gene expression plays a fundamentally important role in maintaining the balance between supply and demand for biomolecules in various organ tissues.

Expression of the glyoxylate cycle genes is also controlled by metabolic status. This was first suggested in cotyledons isolated from germinating castor bean (Lado *et al.*, 1967), where the normal increase in ICL activity at germination was found to be strongly reduced by the presence of 0.1 M glucose. In *Pimpinella* (Anise) suspension cell cultures (Theimer *et al.*, 1981), MS and ICL activity was detected in growth media without sucrose. Addition of sucrose restored the repression of those two enzymes but with different rates. However, further derepression of MS and ICL activity by a further 50% was observed after addition of acetate to the sucrose-free media. These results suggested that acetate shows no effect in the presence of sucrose in the medium and derepression is likely to be dependent on the absence of glycolytic metabolites rather than being a specific effect of acetate.

In cucumber, sucrose repression of the expression of *Ms* and *Icl* in detached and dark-incubated cotyledons has been observed. Furthermore, western blot analysis showed accumulation of MS and ICL enzymes in cucumber protoplasts incubated in mannitol but both enzymes were inhibited in the presence of sucrose. These results suggest that exogenously supplied sucrose can inhibit the accumulation of MS and ICL in both systems thus leading to the hypothesis that sucrose may control the synthesis of these enzymes similar to that observed in bacteria and fungi (McLaughlin and Smith *et al.*, 1994). Graham *et al.* (1994a) used a cell culture system to demonstrate the effect of exogenous and intracellular metabolites on *Ms* and *Icl* gene expression. Both *Icl* and *Ms* expression were coordinately induced in cultures pre-treated with mannitol and this effect was reversed when cells were transferred to media containing either sucrose, glucose, fructose or raffinose. The induction of these genes was closely related to a drop in

these intracellular metabolites below a threshold concentration but not correlated with a decrease in respiration rate. MS and ICL transcripts accumulated in media containing succinate and malate. Under these treatments, respiration rate and intracellular sugars decreased to the same extent as in mannitol treatment. This suggests that intracellular sugar cannot be maintained by gluconeogenesis even though glyoxylate cycle genes encoding MS and ICL are induced. This can be explained by the glyoxylate cycle having an anaplerotic role, whereby it provides succinate to replenish the carbon skeletons of the TCA cycle (Graham *et al.*, 1994a; McLaughlin and Smith, 1994; Kim and Smith, 1994a). Treatment of the cultured cells with 2-deoxyglucose (glucose analog) and mannose, which are phosphorylated by hexokinase but not further metabolised, leads to *Ms* and *Icl* repression. However, the addition of 3-methylglucose that is not phosphorylated resulted in a dramatic increase that was greater than that of mannitol treatment. These observations implicate hexokinase activity in the control of *Ms* and *Icl* gene expression.

Repression by sucrose have also been observed using *Ms* chimeric genes (Graham *et al.*, 1994b). Cucumber protoplasts which contained *Icl* promoter-GUS fusions (Reynolds and Smith, 1995b) were cultured in the dark for 48 h in the presence and absence of sucrose. ICL mRNA and GUS activity were found to be greatly reduced by sucrose. In whole transgenic *Nicotiana plumbaginifolia* seedlings containing *Icl* (Reynolds and Smith, 1995b) or *Ms* (Sarah *et al.*, 1996) promoter-GUS fusions, the induction of GUS activity resulting from dark incubation was repressed by the presence of sucrose.

#### 1.4.2. Carbohydrate abundance and sugar-responsive genes

The expression of several genes is induced by elevation of sugar level. In autotrophic cells, a high level of sugar decreases the expression of photosynthetic genes which allows reallocation of the carbon and nitrogen (otherwise utilised in photosynthetic proteins) to other processes which are favoured by the carbohydrate environment. For example, in the presence of sugar, the nitrate reductase gene in *Nicotiana* leaves (Vincent *et al.*, 1993) and putative SAM synthase gene in leaves of *Lolium temulentum* (Winter *et al.*, 1995) are activated which leads to amino acid synthesis, and turnover of other nitrogen sources.

Genes required for storage reserve synthesis can be up-regulated by sugars. These genes may be similarly affected in both photosynthetic and non-photosynthetic organs (Nakamura *et al.*, 1991; Pena-cortes *et al.*, 1992). These changes may be associated with conversion of chloroplast to either amyloplast (Shaffer *et al.*, 1986) or chromoplast as the sugar level rises (Hutt *et al.*, 1984). Genes for sucrose metabolism are also activated in photosynthetic tissues following manipulations that cause sugar to accumulate (Salanoubatt and Belliard, 1989). These changes result in elevated starch levels (Sonnewald *et al.*, 1994; Sonnewald and Willmitzer, 1992). The expression of several genes for starch biosynthesis is also induced by elevation of sucrose levels. Those encoding ADPG pyrophosphorylase (AGPase), a key step in starch biosynthesis, are markedly sugar responsive in potato (Muller-Robert *et al.*, 1990). However, the increase in AGPase enzyme activity is not so marked and occurs rather slowly.

In maize, the two genes encoding sucrose synthase (*Sh1* and *Sus1*) show different responses to changes in sucrose levels. The *Sus1* mRNA is highly expressed under plentiful sugar supplies whereas reduced level of sugar enhanced *Sh1*. At the protein level, the increase is less pronounced but corresponds to

changes in mRNA levels. However, the contrasting expression of the two maize sucrose synthase genes in response to sugar provides a potential mechanism for altering the distribution of sucrose synthase in response to changing carbohydrate status. Furthermore, it also indicates a complex and carefully controlled regulation of these two isoenzymes (Koch *et al.*, 1992).

Sugar responsive genes are also found among the genes encoding storage proteins. A gene for sporamin storage protein in sweet potatoes (Hattori *et al.*, 1990) is upregulated in stems of plantlets grown *in vitro* on sucrose medium. The expression of a chimaeric gene consisting of a sporamin promoter-CAT (chloromphenicol acetyltransferase) gene preferentially expressed CAT activity in stems of transgenic tobacco plants cultured on a sucrose medium. The patatin storage protein is encoded by a family of genes which also respond positively to high sugar levels (Wenzler *et al.*, 1989). Class 1 patatin is highly tuber-specific in normal potato plants. Expression of class 1 patatin can be induced in stems and petioles of single-leaf stem cutting from which axillary buds have been removed (Paiva *et al.*, 1983) and in leaves incubated in the presence of a high concentration of sucrose (Rocha-Sosa *et al.*, 1989). In addition to that, the vegetative storage protein (*vsp*) gene is also expressed at elevated sugar levels (De wald *et al.*, 1994). Two synergistic regulators, sugar (sucrose, glucose or fructose) and jasmonic acid have been shown to stimulate accumulation of *vsp* mRNA in mature soybean leaves and cell suspension cultures. However, little accumulation occurred when these compounds were supplied separately (Mason *et al.*, 1992). Many of these proteins have enzyme activity in addition to a storage function. For example, the *vsp* gene groups A and B of tobacco encode proteins with phosphatase activity (Sadka *et al.*, 1994) and in soybean vegetative storage protein has lipoxxygenase activity (Grimes *et al.*, 1993).

A group of specific genes which are not active in normal growth responded to various environmental stresses such as wounding when plants are attacked by pests and pathogens (Ryan, 1987). For example, the wound-inducible potato proteinase inhibitor II gene expression is induced by sucrose (Johnson and Ryan, 1990). The expression of a chimaeric potato PI II-CAT gene in transgenic tobacco leaves was enhanced 50-fold when incubated in the presence of 1% sucrose and other carbohydrates such as glucose, fructose and maltose. Furthermore, Kim *et al.* (1991) studied the promoter region of the potato proteinase inhibitor II (PI-II) and identified *cis*-acting regulatory sequences involved in the sugar response using transgenic tobacco plants. In this case, the genetic elements conferring a wound response and the element specifically involved in a sugar response can be distinguished.

#### **1.4.3. Regulation of glyoxylate cycle gene expression**

During postgerminative growth, both *Ms* and *Icl* are shown to be coordinately regulated. The amount of MS and ICL mRNAs increases dramatically during the first 3 days of germination, then decline significantly in green cotyledons and increase again in senescing cotyledons (Kim and Smith, 1994a). The genes encoding glyoxysomal malate dehydrogenase (gMDH) and phosphoenolpyruvate carboxykinase (PEPCK), two other enzymes in the glyoxylate cycle and gluconeogenesis, also show a similar pattern of expression under the same treatment. It has been proposed that the synthesis of ICL and MS is subjected to metabolic regulation in detached leaves and cotyledons (Graham *et al.*, 1992; McLaughlin and Smith, 1994) and cultured cells (Graham *et al.*, 1994b), however this is not the case for gMDH and PEPCK. This suggested that the expression of MS and ICL in germination is not mediated by sugar as in the case

of starvation (Kim and Smith, 1994a,b). It seems that the glyoxylate cycle serves an anaplerotic role rather than gluconeogenic role in starvation conditions.

Further information about the regulation of *Ms* and *Icl* gene expression has been provided by detailed analysis of the *Icl* and *Ms* genes in transgenic plants. A genomic DNA fragment containing the entire cucumber *Ms* coding region including 1856 bases of 5' non-transcribed sequence was transferred into petunia and *Nicotiana*. This resulted in accumulation of mRNA and similar pattern of *Ms* gene expression to that of germination in cucumber (Graham *et al.*, 1990). A comprehensive study of *Ms* expression by its 1076 bp promoter fused to the  $\beta$ -glucuronidase (GUS) gene has been carried out in transgenic seedlings of *Nicotiana* (Graham *et al.*, 1990). The result showed a significant amount of GUS activity thus suggesting that this *cis*-acting element of the cucumber DNA contains sufficient information to direct and regulate faithful expression of the *Ms* gene. Taking these data together, it is demonstrated that the expression of the *Ms* gene in cucumber is maintained when this gene is transferred into *Nicotiana plumbaginifolia*. A 6.5 kb cucumber genomic DNA fragment containing the *Icl* gene was also transferred into *Nicotiana plumbaginifolia* and shown to direct ICL mRNA synthesis in transgenic seedlings upon germination (Reynolds and Smith, 1995b).

Dissecting the *Ms* promoter and analysis of GUS activity allows the *cis*-acting elements mediating sugar and germination to be located (Sarah *et al.*, 1996). The *Ms* promoter containing only 199 bp of sequence upstream from the transcription start is able to direct a significant amount of GUS activity in transformed *Nicotiana* seedlings. However, removal of further 17 bp of the promoter results in almost complete abolition in ability to direct the post-germinative growth response. Complete loss was noted when 40 bp from the resulting 182 bp fragment were deleted to generate a 142 bp fragment. In contrast,



a construct containing 233 bp of the promoter responded to starvation treatment and repression by sucrose, whereas a construct containing 216 bp or less does not direct the starvation response, showing that the gene constructs of 216 and 199 bp fragments are able to direct the germination response but not starvation response. These results indicate that the sequence between 199 and 182 bp is required for the germination response whereas the region between 233 and 216 bp directs the starvation response.

Similar results have been obtained for the *Icl* promoter (Reynolds and Smith, 1995b). Two putative fragments, 2900 and 572 bp were fused to the GUS gene and transferred to *Nicotiana*. Analysis of GUS activity showed that the 572 bp fragment is sufficient to direct the cotyledon-specific and temporal pattern of expression during post-germinative growth, although at a remarkably lower level than the 2900 bp promoter. While the 2900 bp promoter gave rise to significant amount of GUS activity upon starvation and was repressed by sucrose, the 572 bp construct was not starvation responsive. In cucumber protoplasts, promoter deletions of 2900, 2142, and 1663 bp were sucrose responsive but 1142 and 572 bp promoters showed no response. Therefore it is suggested that the promoter between 1663 and 1142 bp is required for the sugar response. It is suggested that the *Icl* promoter contains distinct *cis*-acting elements which direct germination and sugar responses, but the location of the germination response sequences was not established.

## **1.5. Carbohydrate-sensing systems**

Sugars are known to play an important role in the life cycle of prokaryote and eukaryote cells. Beside being important energy sources and structural components, they also control the physiology, metabolism, cell cycle development

and gene expression. In higher plants, sugars affect the growth and development throughout the life cycle from germination to flowering to senescence (Steeves and Sussex, 1989; Brusslan and Thomas, 1992; Graham *et al.*, 1992; Sheen, 1994; Thomas and Rodriguez, 1994). It is becoming apparent now that the expression of genes involved in many essential processes such as photosynthesis, glyoxylate metabolism, respiration, starch and sucrose synthesis and degradation, pathogen defences, wounding response and senescence are controlled by sugars (Chen *et al.*, 1994; Knight and Gray, 1994; Lam *et al.*, 1994; Sheen, 1994; Reynolds and Smith, 1995b).

Several lines of evidence suggest that the sugar effect on gene expression involves specific signalling mechanisms and is not simply the result of their nonspecific effects as substrate for plant growth. Evidence for this is that the effects of sugar are highly selective, in which many genes are not affected. Furthermore, sugars can repress as well as activate responsive genes and in many cases, sugar modulated gene expression can be mimicked by nonmetabolisable sugar analogues (Jang and Sheen, 1994; Sheen, 1990). There are significant differences in the way in which sugar signal transduction pathways are organized in yeast (Trumbly, 1992), mammals (Brun *et al.*, 1993) and plants (Sheen, 1994). However, it has been identified that the first enzyme in the hexose assimilation pathway, hexokinase (HXK), can act as a regulator for sugar-responsive genes in these systems.

The role of hexokinase in sugar sensing was first identified in *Saccharomyces cerevisiae* which expresses two hexokinase genes, *HXK1* and *HXK2* (encoding proteins YH XK1 and YH XK2). In wild-type yeast cells grown on glucose, the expression of *SUC2*, which encoded an invertase capable of hydrolysing di- and trisaccharides such as raffinose, is repressed. However, in *hxx-2* mutants, *SUC2* is not repressed. Glucose analogues such as 2-deoxyglucose (2-

DOG) which can be phosphorylated by YH XK2 but are not further metabolised also repressed *SUC2* expression. Conversely, in *h x k* mutants, 2-DOG is not phosphorylated and so repression does not occur. Taking these data together, it is suggested that YH XK2 has both catalytic and signal transduction activity in regulating gene expression in yeast (Ma and Botstein, 1986).

Hexokinase-mediated sugar sensing in higher plants is however, less well understood compared to yeast because of the complexity that exists in the sugar metabolic pathway. The data that support a regulatory role for hexokinase in plants are based largely on responses to sugars (2-DOG and mannose) that are rapidly phosphorylated by hexokinase but do not readily undergo subsequent metabolism. The evidence of a role for hexokinase as the sugar sensor mediating the repression of genes comes from the study of sugar-regulated expression of genes encoding glyoxylate pathway enzymes (Graham *et al.*, 1994a) and photosynthetic proteins (Jang and Sheen, 1994). In cucumber cell cultures, the presence of glucose, fructose or sucrose results in repression of *Ms* and *Icl* gene expression. Similarly, 2-DOG and mannose, which can be phosphorylated by hexokinase but not further metabolised also resulted in *Ms* and *Icl* repression. Addition of 3-methylglucose, a non-metabolisable glucose analogue that is not phosphorylated, did not result in repression of *Ms* and *Icl* (Graham *et al.*, 1994a). Transient expression studies using a *Cat* reporter gene under the control of the promoters of three photosynthetic genes in maize protoplasts (Jang and Sheen, 1994), have shown repression by various hexoses and 2-DOG while 3-O-methylglucose does not. Furthermore, a competitive inhibitor of hexokinase, mannoheptulose, blocked repression by glucose and 2-DOG. These results indicate that phosphorylation of hexoses by hexokinase appears to be critical for signalling because only the hexose and glucose analogues that can be phosphorylated by hexokinase are effective in triggering sugar-mediated changes in gene expression (Graham *et al.*, 1994a; Jang and Sheen, 1994).

## **1.6. mRNA stability in controlling gene expression**

Regulation of mRNA stability has been demonstrated to be an important determinant controlling gene expression in diverse organisms (Peltz *et al.*, 1991; Gallie, 1993; Green, 1993; Morns *et al.*, 1993; Sachs, 1993). The potential for regulation is obvious in eukaryote systems where a wide diversity of decay rates are displayed, ranging from minutes to days. The high degree of stability of some mRNA species, such as the globin mRNA in erythroid cell (Ross and Pizaaro, 1983) which has a half-life of greater than 24 h, contributes to their accumulation to high steady-state levels. This subsequently leads to sustained and high level synthesis of the encoded protein. Highly unstable species with half-lives of about 10-30 minutes, such as lymphokine and protooncogene mRNAs, are normally present at low steady-state levels (Laird-Offringa, 1992; Greenberg and Belasco, 1993). Although low abundance mRNA could be achieved through a low rate of transcription, the rapid turnover of these mRNAs appears to be important in limiting the synthesis of the encoded protein, and plays a critical regulatory role for brief periods during cellular replication and differentiation. In oat plants, the mRNA encoding the photoreceptor phytochrome has a short half-life of about 1 hour. It is believed to be an important factor in light regulated development (Seeley *et al.*, 1992). Because mRNA degradation is the downstream process, its control could enhance, diminish, or override regulation exerted at the transcriptional level (Ambler and Green, 1996).

### **1.6.1. Mechanism of mRNA degradation in prokaryote cells**

The degradation of most mRNAs is likely to involve the combination of action of endonucleases and exonucleases, with endonuclease cleavage of one or more sites initiating the decay process. (Apirion, 1973; Kernel, 1986; Belasco and

Higgin, 1988). Two 3'-5' exonucleases, polynucleotide phosphorylase (PNPase) and ribonuclease II (RNase II), are especially important for degrading mRNA. The two exonuclease enzymes degrade RNA from the 3' end, removing a nucleotide at a time as they proceed toward the 5'-end of the molecule. The end products of such degradation are the released mononucleotides and a small 5'-terminal oligonucleotide 10 to 20 ribonucleotides long, which is relatively resistant to digestion by either enzyme. An additional enzyme, RNase 1, may complete the process by degrading these short oligonucleotides (Cannistro and Kernell, 1991).

Nevertheless, for the majority of messages, the 3' end is protected from exonucleolytic attack by secondary structure (stem-loops) alone or by the structure together with a stem-loop protein. For such messages, decay is more complex and proceeds by one of several alternative pathways. In some cases, the 3'-5' exonuclease may overcome the blockage presented by the 3' terminal stem-loops and continue processive degradation. For most mRNAs, it appears that this barrier is effectively permanent and further degradation requires the action of endonucleases. Either the terminal structure is removed, exposing a free 3' end and that is accessible to exonucleolytic decay or endonucleolytic cleavage at the 5'-end renders the message susceptible to further cleavage throughout its length (a process that is still not understood), generating fragments with free 3'-ends that are then degraded by 3'-5' processive exonucleases. Even though the action of both endonuclease and 3'-5' exonuclease leads to degradation in the net 3'-5' direction of the *E.coli ompA* mRNA (Melefors and von Gabain, 1988), the concerted action of both enzymes can also lead to more rapid degradation of sequences near the 5' end of an mRNA, causing degradation to proceed in the 'pseudo' 5'-3' direction as occurs with the *Lac z* (Cannistro *et al.*, 1986) and pilin gene (Baga *et al.*, 1988) mRNAs in *E.coli*.

### 1.6.2. Mechanism of mRNA degradation in eukaryote cells

Although degradation often appears to initiate near the mRNA 3' end, mRNA degradation intermediates in eukaryotes remain to be elucidated. The first step in histone H4 mRNA degradation disrupts a 3' stem-loop structure that is likely to be inhibitory to 3'-5' exonuclease, allowing rapid exonucleolytic degradation of the mRNA (Ross *et al.*, 1986). Within minutes after inhibition of DNA synthesis, histone mRNAs shortened at the 3' end by about 5-15 nucleotides were detected by nuclease protection assay. Even though the 5' portion of the mRNA was still intact, the mRNAs were then completely degraded, but no other specific intermediates were detected *in vivo*. It has been shown that the initial intermediate that accumulates *in vivo* is consistent with an initial cleavage of the mRNA in the stem-loop structure. Histone mRNAs are also degraded by a similar pathway *in vitro*. Incubation of polyribosomes containing histone mRNA from a human erythroleukemia cell line resulted in degradation of the histone mRNA by a ribosome-associated nuclease (Ross and Kobs, 1986). The exact pathway of degradation is not known and it is also not clear whether the initial cleavage is endonucleolytic or exonucleolytic. The intermediate terminates in the loop at the 3' end of the mRNA, consistent with an initial endonucleolytic cleavage (Ross *et al.*, 1986). Pure Histone mRNA is degraded 3' to 5' by a nuclease activity that can be extracted from polyribosomes (Peltz *et al.*, 1987; Ross *et al.*, 1987). Thus both the genetic and biochemical evidence supports the conclusion that the initial step in histone mRNA degradation is removal of the 3' end of histone mRNA.

For other mRNAs, such as *c-myc* and *c-fos* mRNAs (unstable mRNA), the degradation process is dependent on protein synthesis (Cochran *et al.*, 1984; Greenberg *et al.*, 1986). A wide range of translational inhibitors that block protein synthesis by different mechanisms were found to lead to a significant stabilisation of these mRNAs. These inhibitors of protein synthesis also block the shutoff of

transcription that occurs following growth factor stimulation of these genes. Under these conditions, inhibition of protein synthesis with cycloheximide increases the half-life of *c-fos* mRNA from ~15 min to several hours (Fort *et al.*, 1987; Wilson and Treisman, 1988).

The requirement of protein synthesis for rapid mRNA decay might be explained in several ways, which are not mutually exclusive. One possibility is that mRNA decay occurs by a mechanism that requires that mRNA messages be actively translated. For example, a ribonuclease that is critical for degrading the mRNA might be associated with the translating ribosome. An alternative explanation of the requirement for protein synthesis is that mRNA degradation may require a labile protein that decays away in the presence of protein synthesis inhibitors (Koeller *et al.*, 1991; Yu *et al.*, 1992). Presumably, if a particular aspect of translation of these mRNA was required for proper decay, different protein synthesis inhibitors might be expected to affect mRNA stability to different extents. In contrast, if a labile protein was involved in the decay process, all translational inhibitors would be expected to have a similar effect on mRNA half-life (Wilson and Treisman, 1988).

In addition to a requirement for protein synthesis in the rapid degradation of *c-fos* mRNA, early studies of *c-fos* mRNA decay also revealed that this message shortens significantly prior to its decay in growth-factor-stimulated cells (Brewer and Ross, 1988; Wilson and Treisman, 1988). Subsequent studies have revealed that this shortening of *c-fos* mRNA occurs at its 3' end and is due to the removal of its poly (A) tail (Wilson and Treisman, 1988; Shyu *et al.*, 1991). The deadenylation of unstable mRNAs prior to their decay may be a mechanism by which many of these messages are degraded, as other mRNAs, including *c-myc* and  $\beta$ -interferon mRNA, also have been shown to undergo deadenylation prior to decay (Laird-offringa, 1991; Peppel and Baglioni, 1991). A careful analysis of the

length of the *c-fos* mRNA poly (A) tail at various times after serum stimulation of NIH 3T3 fibroblasts, indicates that the transcribed portion of the *c-fos* mRNA is stable until the poly (A) is completely removed, whereupon rapid degradation of the mRNA body ensues (Shyu *et al.*, 1991). This finding suggests that deadenylation of *c-fos* and other unstable mRNA is the first step in their decay.

The finding that poly(A) tail removal is followed immediately by degradation of the transcribed portion of *c-fos*, *c-myc* and  $\beta$ -interferon suggest that poly(A) protects these messages from ribonuclease digestion. One simple explanation for how poly(A) could accomplish this is suggested by the discovery in mammalian cell extracts of a ribonuclease activity that degrades RNAs not protected at the 3' end by poly(A) binding protein (PABP) (Berstein *et al.*, 1989). *In vivo*, such enzymes might rapidly degrade unstable mRNA once their poly(A) tails have been shortened so severely as to prevent further binding by PABP. In contrast, long-lived transcripts like  $\beta$ -globulin mRNA, whose poly (A) tail is never shortened to less than 60 nucleotides in transfected fibroblasts, would retain indefinitely their ability to bind PABP at the 3' end and would therefore be resistant to degradation by ribonuclease. These data suggest a similar protection mechanism postulated in eukaryote as in prokaryotes, with the poly (A) tail in conjunction with the PABP taking on the role of the prokaryote stem-loop structure. The absence of detectable *c-fos* or *c-myc* mRNA degradation intermediates that might correspond to the product of endonuclease cleavage in the transcribed portion of these messages suggests that these mRNA may be degraded by a 3' exonuclease after they are deadenylated (Berstein *et al.*, 1989).



### 1.6.3. Mechanism of mRNA degradation in plants

In plants, little is known concerning the mechanism responsible for the alteration of mRNA stability in response to internal or external signals. Study of the plant degradation intermediates is rather limited since the process of transcript degradation is extremely rapid once it is initiated. However, two plant mRNAs, oat PHYA and soybean SRS4, have provided some information on the mechanism of the mRNA decay pathway in higher plants. In both soybean and transgenic petunia, it has been shown that the product of soybean SRS4 (rbcs mRNA) was degraded to a nested set of fragments that are related to each other by increasing deletion from the 3' end of the mRNA. Although most of these RNA fragments retained 5' ends, all of them lacked their poly (A) tails as a result of this degradation. Sequences of the 5' end of these *SRS4* mRNAs were found to be more stable than those at the 3' end of the mRNA. These and other data led to three simple models for RNA degradation in *SRS4* mRNA (Thompson et al., 1992; Higgs and Colbert 1994).

In an exonuclease model, degradation would proceed by a 3'-5' exonuclease whose progress is impeded at specific "hold up points" (Ross and Kobs, 1986). In this model, sequence and structural signals impede the progress of exonuclease to the extent that transient proximal *SRS4* RNA fragments are detectable. The lack of detectable 3' *SRS4* RNA fragment is explained by the nature of nucleolytic action. In the scanning endonuclease model, degradation proceeds by endonuclease cleavage at discrete sites within the mRNA. These cleavage events proceed along the RNA by an unknown mechanism in a 3'-5' direction, removing successively greater portions of the RNA. The nested set of 5' fragments produced are larger and more easily detected than the smaller 3' fragments which are inherently less stable and rapidly degraded by exonuclease. The stochastic model (Tanzer and Meagher, 1995) is different from the other two

models in that the fragments would not be generated in any specific order. In this model, decay is initiated by endonuclease and then the 5' and 3' products are subjected to 3'-5' or 5'-3' exonuclease digestion, respectively.

In the case of *PHYA* mRNA, evidence indicates that fragments of oat *PHYA* mRNA were also observed following degradation, similar to the situation of *SRS4* mRNA. However, unlike the *SRS4* RNA fragments, which accumulate as discrete products, *PHYA* fragments form a continuous distribution that ranges from 4.2 kb to about 200 nucleotides. The analysis of these fragments led to the proposal that degradation involves two pathways with exoribonuclease activities. In this model, 75% of the RNA molecules are degraded by a 5'-3' exonuclease prior to removal of the poly (A) tail whereas 25% of the molecules are deadenylated prior to degradation by the combined action of 5'-3' and 3'-5' exoribonuclease (Higgs and Colbert, 1994).

## **1.7. Factors affecting mRNA stability in higher plants**

### **1.7.1. Hormonal regulation**

In addition to regulating gene expression at the level of transcription, hormonal signals also modulate gene expression by altering mRNA stability or mRNA degradation (Gallie, 1993). In dark-adapted *Lemna gibba*, cytokinin treatment slowed the loss of rubisco small subunit gene (SSU) and light harvesting complex II (LHCP) mRNAs as measured by RNA blot hybridization (Flores and Tobin, 1988). Exposure of *L. gibba* to a pulse of red light following dark incubation of the plants also led to an increase of LHCP mRNA level in response to cytokinin treatment. Nuclear run-on transcription experiments carried out with nuclei isolated from dark-adapted or red light pulsed plants showed at most a 50%

increase in transcription in response to cytokinin. This difference in mRNA accumulation levels may be due to a stabilisation of HLCP transcript in the presence of cytokinin.

Another example of hormonal regulation at the level of mRNA stability derives from the studies of ethylene regulation of gene expression in ripening tomato fruit. It has been shown that ethylene induces expression of several genes in this system, in most cases at the transcriptional levels. In at least one case, however, the steady-state mRNA level for the *E17* gene (subsequently called ethylene-responsive proteinase inhibitor) increases by six fold with no apparent increase in the rate of transcription (Margossian *et al.*, 1988; Lincoln and Fisher, 1988).

Absciscic acid (ABA) is found to down regulate  $\alpha$ -amylase mRNA. Early reports indicated ABA-mediated repression of  $\alpha$ -amylase mRNA translation during germination. However, subsequent work focusing on two main classes of  $\alpha$ -amylase, (high *pI* and low *pI*) genes individually showed that the addition of ABA resulted in the degradation of the stable high *pI*  $\alpha$ -amylase mRNA but not the low *pI* form (Nolan *et al.*, 1987; Nolan and Ho, 1988). Inhibition of transcription or translation prevented the ABA-mediated  $\alpha$ -amylase mRNA turnover, demonstrating that ongoing gene expression is required for high *pI* mRNA degradation. ABA can also act as a positive regulator at the posttranscriptional level. The ABA-inducible *EM* gene from wheat enhances reporter gene expression by 10 fold in rice protoplasts in the absence of ABA but increases another two-fold when the hormone is present (Marcotte *et al.*, 1989). As the rate of *Em* protein synthesis follows *Em* mRNA levels, ABA may regulate *Em* mRNA stability (Williamson and Quatrano, 1988).

### 1.7.2. Heat shock regulation

Heat shock is another stimulus known to affect the expression of many genes (Vierling, 1991). The response involves dramatic changes at the transcriptional and posttranscriptional levels. Following heat shock, normal cellular messages are released from polysomes to be replaced by heat shock specific mRNAs (Storti *et al.*, 1980).

In heat-shocked aleurone tissue,  $\alpha$ -amylase transcripts are unstable, whereas under normal conditions, this is a stable message (Belanger *et al.*, 1986). The mRNA is not sequestered but appears to have a maximum half-life of 30 minutes when the incubation temperature is raised from 25°C to 40°C. It has been suggested that the short half-life of the transcript during heat shock is due to active destabilisation of otherwise stable mRNA (Brodl *et al.*, 1991). The mRNA for two other secreted proteins, a thiol endoprotease and endochitinase, were also rapidly degraded with the onset of heat shock, although the degradation rate of nonsecretory protein message, such as actin and  $\beta$ -tubulin mRNAs, remained unaffected (Brodl *et al.*, 1991). As heat-shock normally induced disruption of the endoplasmic reticulum in this cell type (Belanger *et al.*, 1986), those messages whose products are co-translationally transported across the ER (secretory proteins), may be targeted for rapid turnover to ensure full repression of this class of protein. These data support the hypothesis that heat-shock induced disruption of the endoplasmic reticulum (ER) leads to the rapid decay of mRNA that is normally translated on ER-bound polysomes.

### 1.7.3. Light regulation

Light effects on gene expression both at the transcriptional and posttranscriptional levels have been studied extensively. It is most likely that light affects the stability of some light-responsive mRNAs. The rubisco small subunit gene (*SSU*) is subject to a posttranscriptional control mechanism. When petunia plants are placed in darkness, the level of *rbcs* transcript drops in parallel to the decrease in transcription, suggesting a lack of posttranscriptional control under these conditions (Thompson and Meagher, 1990). However, the level of *rbcs* mRNA increases to a higher level after re-exposing the petunia plants to light following 48 hour in darkness implicating a stabilisation of mRNA in light (Thompson and Meagher, 1990).

In potato, *rbcs* transcripts decay more rapidly in the dark than in the light in the presence of cordycepin. This indicates that the transcripts are stabilised in the light or destabilized in the dark (Fritz *et al.*, 1991). It has been shown that stability of *rbcs* mRNAs is also modulated by light in mature soya bean (Shirley and Meagher, 1990). The discrepancies between mRNA accumulation levels and transcription rates suggest that *rbcs* transcripts are less stable in the dark than in the light (Thompson and Meagher, 1990). Conversely, when soya bean seedlings were shifted to dark, *rbcs* gene transcription decreased 32-fold whereas mRNA accumulation level decreases by only eight-fold suggesting that *rbcs* mRNA is four times more stable in the dark than in the light (Shirley and Meagher, 1990). Other genes regulated posttranscriptionally by light include the catalase subunit genes from cotton. SU2 mRNA accumulation is light dependent but not SU1 mRNA. Both of these genes are transcribed actively throughout postgerminative stages, but SU1 mRNA levels drop significantly which suggest selective destabilisation (Ni and Trelease, 1991). In dark-grown maize leaf tissue, the Cat2 mRNA cannot be translated *in vitro* due to possible modification that renders it incompetent for

translation (Skadsen and Scandalios, 1987). Phytochrome (A) (phyA) mRNA abundance decreased rapidly in total mRNA samples in etiolated oat seedlings following a red light pulse.

The rate of transcription by nuclear run-on is not parallel with the increase in transcript accumulation (Dickey *et al.*, 1992). This fact is clearly shown by observation of two light-responsive genes, *fed-1* from pea (Dickey *et al.*, 1992) and *fed A* from *Arabidopsis* (Vost *et al.*, 1993). Pea ferredoxin 1 (*fed-1*) mRNA levels increased in the presence of light under the direction of various chimeric reporter genes in mature leaves of transgenic tobacco (Elliot *et al.*, 1989). These experiments showed that a 230 nucleotide *fed-1* fragment consisting of the 5'-untranslated region (5'-UTR) is necessary for the light response as well as a region within the first third of the *fed-1* open reading frame fused to reporter sequences (Dickey *et al.*, 1992). Blocking translation by insertion of a nonsense codon into the chimeric gene transcript abolishes the light responses.

Furthermore, the rate of transcriptional initiation remains unchanged by isolated nuclei. Together, these data demonstrate the requirement of the light-responsive element within the transcribed region for stabilising *fed-1* mRNA in light or destabilising *fed-1* in the dark. In *Arabidopsis*, *fed A* transcript in light is twenty time higher than in the dark, whereas the transcription rate was only twofold higher in the light than in the dark. The large difference between mRNA accumulation and transcriptional activity suggests that the *fed A* transcript is either stabilised in the light or destabilised in the dark (Vorst *et al.*, 1993).

## **1.8. Transformation systems in higher plants**

### **1.8.1. Gene transfer systems**

Gene transfer systems have had a major impact on the investigation of the regulatory mechanism of plant development. For instance, transgenic plants serve as analytical tools to describe metabolic pathways, to study *cis*-acting and *trans*-acting factors that control gene function, and to understand the mechanism of plant response to environmental stresses. Mobile genetic elements (transposon) and T-DNA can serve as insertional mutagens, allowing the identification and isolation of coding and regulatory sequences of plant genes.

### **1.8.2. DNA delivery methods**

Transformation techniques for plants provide the means to allow nucleic acids to pass the cell wall, plasma membrane and nuclear envelope without affecting the viability of the target cell. Extensive efforts have been made to develop systems for the delivery of DNA into plant cells, including particle bombardment (Cristou, 1992), protoplast transformation via polyethylene glycol or electroporation (review, Potrykus I, 1991), microinjection of DNA into mesophyll protoplasts (Crossway *et al.*, 1986) and macroinjection of DNA into young floral tillers of cereal plants (de la Pena *et al.*, 1987). Among these methods, the most frequently used strategy for generating transgenic plants is the biological transfer system which employs *A. tumefaciens* or *A. rhizogenes* (for review see Potrykus I, 1991).

### 1.8.3. Hairy roots

In cucumber, the studies of *Ms* and *Icl* gene expression have been limited to the use of heterologous transgenic *Nicotiana* plant (Graham *et al.*, 1990; Reynolds and Smith, 1995b; Sarah *et al.*, 1996) and electroporated protoplasts of cucumber (Graham *et al.*, 1994; Reynolds and Smith, 1995b). The ideal would be to use a homologous transgenic cucumber plant but this is difficult to produce. So in this project, a more promising system involving the culture of roots obtained by transformation with *A. rhizogenes* was adopted. These hairy root cultures have the same metabolic features as normal root cultures, yet are rapid-growing adventitious roots produced from the infection site. The induction of morphological changes is preceded by the stable integration of a portion of the Ri (root-inducing) plasmid into the plant genome, a characteristic it shares with the causal agent of the crown gall tumour disease, *A. tumefaciens* (Chilton *et al.*, 1982). The biochemical and molecular basis of hairy root phenotype is less understood than that of the 'crown gall' caused by *A. tumefaciens*, but the genes encoded in the Ri T-DNA (transfer-DNA) also appear to alter the balance of and tissue sensitivity to phytohormones (Schmulling *et al.*, 1988). Hairy root cultures offer an intrinsic high stability (Aird *et al.*, 1988; Inomata *et al.*, 1993), growing rapidly *in vitro*. Furthermore, the possibility of establishing and optimising the growth of hairy root cultures in laboratory scale pilot plant bioreactors seems to be a realistic prospect (Hilton *et al.*, 1988; Savary and Flores, 1994). Hairy roots produced from cucumber have previously been reported (Trulson *et al.*, 1986)

## 1.9. Aims of the thesis

*Ms* and *Icl* genes have been isolated and characterized from several plant species such as *Brassica napus*, (Comai *et al.*, 1992; Zhang *et al.*, 1993), and



cucumber (Graham *et al.*, 1989; Reynolds and Smith, 1995a). The studies of their expression throughout the defined stages of plant development and under the control of metabolic status have provided information to understand more about the factors as well as the mechanisms that control expression. The coordinate regulation of these genes has been reported in many plant species suggesting common factors might exist in regulating both genes. However, distinct *cis*-acting elements appear to be necessary for induction of *Ms* and *Icl* genes at different stages of plant development as well as for exogenous stimuli. In cucumber plants, as discussed in the Chapter 1, deletion analysis of *Ms* (Sarah *et al.*, 1996; Graham *et al.*, 1994b) and *Icl* (Reynolds and Smith, 1995b) promoters has identified different regions of the genes necessary to direct postgerminative and sugar responses using transgenic *Nicotiana* and transient expression in electroporated protoplasts of cucumber.

*Ms* and *Icl* gene expression in cucumber is also shown to be induced in a variety of tissues such as in cotyledons, senescing leaves and petals of cucumber. (McLaughlin and Smith, 1994; Graham *et al.*, 1992) suggesting that the synthesis of ICL and MS is not tissue specific or confined only to the fat-storing tissue of germinating oilseeds, but rather that the physiological or metabolic state may control the expression of these genes. The most convincing evidence that metabolites may play some part in the control of *Icl* and *Ms* gene expression in higher plant comes from the studies using *Pimpinella anise* suspension cultures. These studies have demonstrated that removal of sucrose from the growth medium resulted in the induction of MS and ICL, and was subsequently enhanced by the addition of acetate into the medium. Furthermore, the most important observation on culture cells, protoplasts (Graham *et al.*, 1994a/b) and detached cotyledons (McLaughlin and Smith, 1994) has suggested that *Ms* and *Icl* gene expression may be modulated by certain metabolites. As indicated earlier, studies of *Ms* and *Icl* transgenes are confined to the use of *Nicotiana* heterologous expression systems

and cucumber protoplasts which are not necessarily reliable and reproducible. A more valuable system would involve transgenic cucumber plants but this is difficult to achieve. In this work, stably-transformed hairy roots of cucumber have successfully been obtained using *Agrobacterium rhizogenes*. Previous work by Graham *et al.* (1992), has shown that the *Ms* gene was expressed in roots when they were excised from the plant and incubated in the dark for several days. Based on these data, it is suggested that genetically transformed cucumber hairy roots could be a potential experimental system for the manipulation of *Ms* and *Icl* gene expression. In particular, it is the primary aim of this project to study *Ms* and *Icl* gene expression using transgenic hairy roots. This would involve the use of a series of *Ms* and *Icl* promoters which would enable subsequent comparison of homologous gene expression of hairy roots with that of the heterologous and transient expression, leading to the confirmation and identification of regulatory elements necessary for the expression of these genes during plant development and in response to metabolite status.

The control of gene expression can also occur at the posttranscriptional level. Among these processes is the control of mRNA stability. Data obtained so far have shown that mRNA degradation has a profound impact on gene expression and the rate of mRNA decay can be modulated in response to environmental and development signals. For example, in the  $\alpha$ -amylase gene expression in suspension-cultured cells, both transcription and mRNA stability have been shown to affect its steady-state level under starvation (Sheu *et al.*, 1994). On the contrary, the effects of sugar on MS and ICL mRNA levels have so far been well documented elsewhere but how it affects MS and ICL mRNA stability is yet to be elucidated. Thus, part of this project focuses on the effect of sucrose on mRNA stability in order to answer this question.

## **CHAPTER TWO**

### **MATERIALS AND METHODS**

## Chapter 2: Materials and Methods

### 2.1 Biological Materials

#### 2.1.1 Plant material

Cucumber seeds used throughout this study were *Cucumis sativus* L. cv Marketmore. These seeds were purchased from W.K. McNair Portobello, Edinburgh. For *Agrobacterium rhizogenes* transformation of cucumber hypocotyls, seeds were sterilised in 70% (v/v) ethanol for 1 min followed by 10% Chlorox (commercial bleach containing 5.25% w/v sodium hypochlorite) for 30 min. Seeds were then rinsed in sterile water, followed by soaking in 25 µg/ml rifampicin for 2 h and then placed on Murashige and Skoog (M&S) medium containing 1% (w/v) sucrose and 0.8% (w/v) agar for 3 days in the dark and followed by 2 days in the light.

Hypocotyls were excised for transformation with *Agrobacterium rhizogenes*. For detached root experiments, seeds were imbibed overnight at 4°C in sterile distilled water, then sown on two layers of moist filter paper. The seedlings were maintained in a growth room with 12 h per day lighting and a constant temperature of 25°C for 5 days. After 5 days, root tips (terminal 1-2 cm) were excised and incubated on moist filter papers for up to 6 days at 25°C in the dark. For defoliation and shading experiments, seedlings were grown in vermiculite for 10 days, in a cycle of 10 h light, 14 h dark at 25°C. They were then transferred to 20% Hoaglands No. 2 basal salt solution (Sigma Co.) for a further 10 days. Plants were then subjected to defoliation or shading treatments and roots collected for analysis 6 days later.



2.1.2. Bacterial strains and their genotypes

*Escherichia coli*:

XLI-Blue            *supE44 hsdR17 recA1 endA1gyrA46 thi relA1 lacF'*[*proAB*<sup>+</sup>*lacI*<sup>f</sup>  
*lac ZM15 Tn 10(te<sup>tr</sup>)*] (Bullock *et al.*, 1987)  
Used as a host for recombinant manipulation.

*Agrobacterium rhizogenes*:

Strain A4:            Binary vector host strain (Tepfer, 1990)

*Agrobacterium tumefaciens*:

LBA 4404:            Genotype not available. Carries a cryptic and a ‘disarmed’ Ti  
plasmid , the latter lacking the entire T-DNA, but with intact *vir*  
region. The bacterial chromosomes carries streptomycin resistance  
and disarmed Ti plasmid carries rifampicin resistance (Hoekema  
*et al.*, 1983).

2.1.3. Bacterial plasmids

Table 2.1 Sources and description of plasmids

Plasmid	Source
pBIN19	Binary vector used for plant transformation (Bevan 1984).
pBSICL1.4	A partial cDNA clone encoding cucumber isocitrate lyase ( Reynold and Smith, 1995a).
pBSMS1.9	A full length cDNA encoding cucumber malate synthase (Graham <i>et al.</i> , 1989).
pBSH18	A full length cDNA clone encoding cucumber NADH-dependent hydroxypyruvate reductase (Greeler <i>et al.</i> , 1989).
PBS/1539	A partial cDNA clone encoding a small subunit of ribulose-1,5- biphosphate carboxylase oxygenase from cucumber (Greenland <i>et</i> <i>al.</i> , 1987).

## **2.2. Miscellaneous**

### **2.2.1. Glassware and plasticware**

Glassware and plasticware used in experiments involving nucleic acids were autoclaved at 121°C, 15p.s.i. for 30 min. For sequencing procedures, siliconisation was carried out by rinsing surfaces of glassware with 'repelcote' water repellent, then inverting on a few layers of paper towel and left to dry in a fume hood. After siliconising, glassware was thoroughly rinsed with distilled water. For work involving RNA samples, glassware was treated with 0.01% diethyl pyrocarbonate (DEPC), incubated overnight at 65°C, followed by autoclaving.

### **2.2.2. Chemical and biological reagents**

Unless otherwise stated, all chemicals and reagents were purchased from Sigma Chemical Co. Ltd., and BDH, and were analytical grade (ANALAR).

Restriction endonucleases and DNA modification enzymes were purchased from Northumbria Biologicals Ltd. (NBL), Boehringer Mannheim Biochemical GmbH, Pharmacia LRB and Gibco-BRL.

### **2.2.3. Bacteriological media**

Luria-Bertani (LB) medium ( 1 litre) : 10 g bacto-tryptone (Difco Laboratories)  
5 g bacto-yeast extract (Difco)  
10 g NaCl . Adjust pH to 7.0 with  
5 M NaOH prior to autoclaving.

LB agar : As for Luria-Bertani medium, but with the addition of 15 g/L agar prior to autoclaving.

2 X YT medium (1 litre) : 16 g bacto-tryptone  
10 g bacto-yeast extract  
5 g NaCl. Adjust pH to 7.0 with 5.0 M NaOH prior to autoclaving.

NA (Nutrient agar) : NA was purchased from Difco and reconstituted according to the manufacturers instructions.

#### **2.2.4. Plant tissue culture media**

All media were based on the base medium of Murashige and Skoog (1962) (M&S medium, Flow Laboratories, Irvine).

Shooting medium (1 litre) : 4.7 g M&S medium, 10 g sucrose, 0.2 mg N<sup>6</sup>Furfurylaminopurine (kinetin), 2 mg naphthaleneaceticacid. (NAA), 8 g bacto-agar , pH 5.7.

Expansion medium (1 litre) : 2.35 g M&S medium, 5 g sucrose, 8 g bacto-agar pH 5.8

Rooting medium (1litre) : 2.35 g M&S medium, 8 g bacto-agar.

Germination medium (1litre) : 4.7 g M&S medium, 10 g sucrose, 10 ml 5% (2-[N-Morpholino]ethanesulphonic acid) MES pH 5.7.

**2.2.5. Growth, maintenance and storage of bacterial cultures**

Using standard sterile techniques, a single bacterial colony was picked using a wire loop and used to inoculate aliquots of bacterial media (LB) containing the required concentration of antibiotic. Cultures were grown at 37°C (*E.coli*) or 30°C (*Agrobacterium rhizogenes*) with constant agitation. For isolation of a single bacterial colony by streaking out, LB agar was used ( LB with the addition of 15 g/l bacto agar) containing appropriate antibiotic. Bacteria were stored for periods of up to three weeks at 4°C on sealed agar plates.

Storage of bacteria for indefinite periods of time was achieved by mixing 750 µl of an overnight bacterial culture grown in LB with an equal volume of 40% (v/v) glycerol in LB in an eppendorf tube and flash- freezing in liquid nitrogen. The cultures thus treated were stored indefinitely at -80°C. When such cultures were required for use, an inoculum was obtained by scraping a sterile tooth pick over the surface of the culture and growing up overnight in 5 ml 2 X YT with antibiotic selection. This reactivated culture was subsequently used as a starter innoculum for larger volumes.

**2.2.6. Antibiotics used for bacterial selection**

Antibiotic	Selective concentration for		Stock (mg/ml)	Solvent
	<i>E.coli</i> (µg/ml)	<i>A. rhizogenes</i> (µg/ml)		
Ampicillin	50	-	100	water
Kanamycin	100	100	100	water
Rifampicin		100	100	methanol
Streptomycin	-	500	300	water



Tetracyclin	10	-	10	ethanol
Vancomycin			500	water

### 2.2.7. Radiochemicals

$\alpha$ -[ $^{32}\text{P}$ ]dCTP(3000 Ci mmol $^{-1}$ ) and  $\alpha$ -[ $^{35}\text{S}$ ]dCTP(>5000 Ci mmol $^{-1}$ ) were purchased from Amersham International plc.

### 2.2.8. Autoradiography film

RNA filters were covered in Saran wrap and exposed to Cronex 4 X-ray film for the required length of time in sealed cassettes at -70°C for detection of  $^{32}\text{P}$ -labelled nucleotides.

Sequencing gels were placed directly onto Cronex 4 X-ray film in a sealed cassette and incubated for the required length of time at room temperature for detection of  $^{35}\text{S}$ -labelled nucleotide. X-ray films exposed as above were developed using an X-Omat developer.

## 2.3. Molecular Biology Methods

### 2.3.1 DNA isolation and analysis

#### 2.3.1.1 Small scale plasmid isolation

Recovery of plasmid DNA from 1-1.5 ml cultures of bacteria was carried out essentially as described in Sambrook *et al.* (1989). A single colony was inoculated in 5 ml LB containing the appropriate antibiotic and incubated for 12-

16 h at 37°C with constant shaking. 1.5 ml of the culture was centrifuged at top speed for 30 sec in a microcentrifuge tube to pellet the bacterial cells. After removing the supernatant by aspiration, cells were thoroughly resuspended in 100 µl of solution I (50mM glucose, 25mM Tris-Cl, pH 8.0, 10 mM EDTA pH 8.0) and incubated on ice for 5 min. Then 200 µl of freshly prepared solution II (0.2 M NaOH, 1% SDS) were added and the contents of the tube were gently mixed by inversion to lyse the cells, followed by 5 min incubation in ice.

Following addition of 150 µl of solution III (3 M potassium acetate, 2 M acetic acid) the tube was gently shaken to ensure adequate mixing and subjected to centrifugation at top speed in a bench top centrifuge for 5 min. An equal volume of phenol/chloroform (1:1) was added to the supernatant and vortexed vigorously for a few seconds, then centrifuged for 10 min at 4°C. Nucleic acid was precipitated from the upper aqueous phase by the addition of 2.5 volumes of ice-cold ethanol and incubated at -20°C for 20 min. Nucleic acids were then pelleted by centrifugation at 4°C for 30 min. The pellet was then washed in 70% (v/v) ethanol, dried in a Howe Gyro-vap (V.A.Howe and Co. Ltd, Banbury, UK.) and resuspended in 20-30 µl sterile distilled water. 2-3 µl of this final suspension were routinely used in restriction digests. Since this method does not eliminate RNA, digests were routinely carried out in the presence of 0.1 vol. of 0.5 mg/ml RNase.

#### **2.3.1.2 Large scale plasmid isolation**

Large scale plasmid preparations were routinely carried out from 500 ml bacterial cultures according to the methods of Sambrook *et al* (1989). For the extraction of smaller volumes this procedure was scaled down to the appropriate volumes but otherwise remained essentially as described below.

500 ml of an overnight culture (stationary phase) was spun down in a Sorvall RC-5B centrifuge at 10,000 rpm for 10 min at room temperature. After pouring off the supernatant, the bacterial pellet was resuspended in 20 ml of solution I, 40 ml solution II added to lyse the cells, and genomic DNA and protein precipitated by the addition of 30 ml of solution III by a gentle yet thorough agitation of the mixture. A crude nucleic acid preparation was obtained by centrifuging this mixture at 10,000 rpm for 5 min, and filtration of the supernatant through polyallomer wool. Nucleic acids were precipitated by the addition of an equal volume of propan-2-ol to the filtrate and a further centrifugation at 10,000 rpm for 5 min. After resuspension of the nucleic acid pellet in 3 ml sterile distilled water, 3 ml of 10 M LiCl were added in order to precipitate contaminating RNA which was then removed by centrifugation at 10,000 rpm for 10 min. The supernatant was transferred to a fresh tube and plasmid precipitated using 1 volume of propan-2-ol.

Following incubation at room temperature for 5 min, the plasmid was recovered by centrifugation at 10,000 rpm for 5 min, dried thoroughly in a vacuum dessicator, resuspended in 500 µl sterile distilled water and transferred to a 1.5 ml Eppendorf tube. After treating with RNase to remove any remaining contaminant RNA molecules, high molecular weight nucleic acids were precipitated with an equal volume of 1.6 M NaCl (w/v), 13% PEG 6000 and removed by centrifugation at top speed in a microcentrifuge for 10 min at 4°C. The resulting pellet was resuspended in 200 µl sterile distilled water and phenol/chloroform extracted twice. Plasmid DNA was finally precipitated with 10 µl ammonium acetate (10 M) and 2.5 volumes ethanol, pelleted by centrifugation, washed in 70% ethanol, dried and resuspended in 200 µl water. Nucleic acid concentration was estimated by spectrophotometric analysis.

### **2.3.1.3. *Agrobacterium* DNA isolation**

*Agrobacterium* DNA isolation techniques were based on an alkaline lysis procedure and allows the DNA structure of many transformants to be verified quite rapidly. A small amount of helper Ri-plasmid will be purified, but it does not interfere with binary vector analysis.

*Agrobacterium* cells were grown 48 h in 5 ml LB medium containing the appropriate concentration of antibiotic with vigorous shaking at 28°C. Bacterial cells were pelleted by centrifugation and resuspended in 100 µl of ice-cold solution I, followed by incubation at room temperature for 10 min. 0.2 ml of freshly prepared solution II were added and the sample shaken to mix. 30 ml phenol equilibrated with two volumes of solution II was added and shaken until viscous. 150 µl of 3.0 M sodium acetate pH 4.8 was added and sample was incubated at -20°C for 15 min. The two phases were separated by centrifugation at 10,000 rpm for 5 min. DNA was precipitated by the addition of sodium acetate to a final concentration of 0.3 M, and 2 volumes cold ethanol, and storage at -20°C for 2 h. The DNA was pelleted by centrifugation at 10,000 rpm at 4°C washed in 70% (v/v) ethanol, briefly dried in a vacuum dessicator and resuspended in 50 µl sterile distilled water.

### **2.3.1.4 Estimation of DNA and RNA concentration**

The concentration of plasmid DNA and RNA were determined using absorbance properties of nucleic acids. Both molecules absorb light at a wavelength of approximately 260 nm, while contaminants will shift the absorbance peak from the wavelength. For the purposes of spectrophotometer analysis, a Beckman DU-64 spectrophotometer was routinely used. Samples were

scanned over a wavelength range from 220-320 nm and nucleic acid concentration determined by measurement of the absorbance at 260 nm . The ratio of the  $A_{260}$  to that at  $A_{280}$  gave an estimation of the purity of the samples. For RNA, 1  $A_{260}$  unit is equivalent to 40  $\mu\text{g/ml}$  and for DNA, 1  $A_{260}$  is equivalent to 50  $\mu\text{g/ml}$ .

#### **2.3.1.5. Restriction digests**

Endonuclease digestion of plasmid DNA was routinely carried out using a 10-fold excess of enzyme. Reaction mixes containing the DNA to be digested, the relevant endonuclease (no more than 1/10th the final reaction volume) and 1/10th volume of the appropriate buffer supplied with the enzyme were incubated according to the manufacturers instruction for generally, 1-2 h. When carrying out a double digest i.e., digesting with different restriction enzymes, the enzymes were diluted in the reaction mix such that the combined enzyme volume did not exceed 1/10th the final reaction volume. The choice of buffer was such that each enzyme was capable of at least 50% activity. Where this was not possible, the DNA was cleaved using one enzyme first, then extracted with phenol-chloroform followed by an ethanol precipitation of the upper aqueous phase. The resultant pellet was resuspended in an appropriate volume of sterile distilled water and treated with the second restriction enzyme.

#### **2.3.1.6. Horizontal gel electrophoresis of DNA**

Samples were routinely analysed by electrophoresis in agarose gels of 0.7-1.7 % (w/v) agarose prepared in 1 X TAE (0.004 M Tris-Acetate, 1 mM EDTA, pH. 8.0). The concentration of the agarose chosen was dependent on the size of the fragment being electrophoresed. Gels were run at 100-150 V in 1 X TAE buffer.

Prior to electrophoresis 1/5th volumes of 6X DNA gel-loading buffer (0.25%(w/v) bromophenol blue, 0.25%(w/v) xylene cyanol FF, 30% (v/v) glycerol in water) was added to the samples. DNA size standards (1 kbp ladder, Boehringer Mannheim) were run alongside the samples in order to facilitate size determination of the sample bands upon visualisation using a short wavelength trans-illuminator (Hybaid).

## **2.3.2. RNA isolation and analysis**

### **2.3.2.1. Total RNA isolation from cucumber roots**

Plant material was ground into a fine powder with liquid nitrogen using a baked pestle and mortar. The frozen powder was immediately transferred to a pre-chilled 15 ml corex tube to which a minimum of 4 ml extraction buffer (100 mM Tris-HCl, 6% (w/v) Para-aminosalicylic acid, 1% (w/v) tris-isopropylphenylmethane). The tube was then vortexed for 10 sec.

After centrifugation at 10,000 rpm for 10 min at 4°C, the supernatant was removed to a fresh tube followed by the addition of an equal volume of phenol:chloroform:iso-amyl alcohol (25:24:1 by volume). The tube was vortexed and then centrifuged for 15 min at 10,000 rpm. The aqueous phase was removed to a fresh tube and the extraction step repeated (depending on the plant tissue, in order to get clear aqueous phase without contaminants). After final extraction, the upper aqueous phase was removed to a fresh tube and nucleic acids were precipitated with 2.5 volumes of ethanol and 1/10th volume of 3.0 M Na Acetate (pH 4.8) at - 20°C overnight. Then the nucleic acid was pelleted by centrifugation at 10,000 rpm for 10 min at 0°C, washed in 70% (v/v) ethanol and resuspended in 1 ml DEPC-treated double distilled water (0.01% v/v). Total RNA was selectively

precipitated by the addition of an equal volume of 5 M LiCl to a final concentration of 2.5 M and incubated at 4°C for 12-16 h. Total RNA was collected by centrifugation as above and precipitation by addition of ethanol repeated. Recovered RNA was washed twice in 70 % (v/v) ethanol, dried and resuspended in DEPC-treated sterile double distilled water. RNA concentration was estimated in a Beckman spectrophotometer at 260 nm and 280 nm.

#### **2.3.2.2. Horizontal gel electrophoresis of RNA**

RNA was fractionated on horizontal, denaturing 1.5% (w/v) agarose gels made up by dissolving 1.5 g agarose in a solution containing 2 ml 50 X MOPS in 81 ml water. Once the agarose has been dissolved completely, the mixture was allowed to cool 50°C before adding 17 ml of a 37% (v/v) solution of formaldehyde and pouring the gel immediately. Electrophoresis was carried out in 1 X MOPS (3-[N-morpholino] propanesulphonic acid, 20 mM MOPS, 5 mM sodium acetate pH 7.0, 1 mM EDTA pH 8.0). Samples were prepared for electrophoresis by desiccating aliquots to complete dryness under vacuum and redissolving the RNA in 15 µl of 1 X RNA gel loading buffer (1 X MOPS buffer, 50% [v/v] formamide, 6.6% [w/v] formaldehyde, 3% [w/v] Ficoll 400, 0.02% [w/v] bromophenol blue, 50 µg/ml ethidium bromide). Samples were heated at 65°C for 5 min immediately prior to loading. This RNA was visualised using a short wavelength trans-illuminator (Hybaid).

#### **2.3.2.3. Northern blot analysis**

RNA gels did not require any further treatment following electrophoresis and so could be blotted directly to Hybond-N-filter (Amersham, UK) using

capillary action according to the method of Southern (1975). Transfer was facilitated by imposing a salt gradient across the gel from a reservoir of 20 X SSPE (3.6 M NaCl, 0.2 M sodium dihydrogen orthophosphate pH 7.7, 20 mM EDTA pH 8.0) to the filter which had been soaked in 2 X SPPE. After transfer, the filters were rinsed in 2X SSPE baked at 60°C for 15 min and the RNA was crosslinked to the membrane by ultra-violet irradiation at 0.4 J cm<sup>-2</sup> using a Hybaid crosslinker.

### **2.3.3. Generation of radiolabelled probes**

#### **2.3.3.1 Oligolabelling of double-stranded DNA fragments**

The method of Feinberg and Vogelstein (1984) was employed in the generation of  $\alpha$ -[<sup>32</sup>P]dCTP-labelled double stranded DNA probes.

5 X Oligolabelling buffer - 250 mM Tris-Cl pH 8.0

25 mM MgCl<sub>2</sub>

5 mM  $\beta$ -mercaptoethanol

2 mM each dATP, dGTP, dTTP

1 M HEPES (4-(2-hydroxyethyl)-piperazine-1-ethanesulphonic acid (adjust pH to 6.6 with 4 M NaOH).

20-50 ng of the DNA fragment was denatured by heating in a heating block at 100°C for 5 min and then cooled on ice. To the denatured DNA was added:

5 X oligo labelling buffer (OLB)	10 $\mu$ l
Bovine serum albumin (10 mg/ml)	2 $\mu$ l
[ $\alpha$ - <sup>32</sup> P] dCTP (30 $\mu$ Ci)	3 $\mu$ l



DNA polymerase I Klenow fragment	2 $\mu$ l
Deionised water	to 50 $\mu$ l

The labelling reaction mixture was incubated at room temperature overnight and terminated by the addition of 200  $\mu$ l of STOP buffer (50 mM Tris-Cl, pH 7.5, 50 mM NaCl, 5 mM EDTA pH 8.0, 0.5% [w/v] SDS). Incorporation of over 80% was generally obtained. Unincorporated nucleotides were removed by centrifugation through a sephadex G-50 (pharmacia) column equilibrated with TE. The column was prepared using a 1 ml syringe plugged with siliconised glass wool. This was filled with sephadex G-50 and centrifuged at 2500 rpm in a bench top centrifuge for 1.5 min. The radiolabelled DNA was collected in a microcentrifuge tube and stored at -20 °C until required. The probe was denatured by boiling before adding to the hybridisation buffer.

#### **2.3.3.2. Hybridisation of radiolabelled probe to membrane-bound RNA**

After UV crosslinking, the membrane was incubated in a glass tube in a rotisserie oven (Hybaid) for a minimum of 2 h at 65°C in prehybridisation solution (4X SSPE, 20 mM Tris-Cl pH 7.6, 1.0% [w/v] SDS, 2 X Denhart's solution, (1% [w/v] BSA fraction V, 1% [w/v] Ficoll 400, 1% [w/v] polyvinylpyrrolidone [ $M_r$  40000] ), 10% (w/v) dextran sulfate). This step will block sites on the membrane to which probe may potentially bind in a non-specific manner.

After sufficient time had elapsed, the solution was replaced with fresh solution, the denatured radiolabelled probe was added after first boiling for 5 min and hybridisation allowed to progress overnight at 65°C. After hybridisation, the membranes were routinely washed in progressively more stringent (lower salt concentration) washes as follows:

<b>Wash</b>	<b>Solution</b>	<b>Condition</b>
Wash I	4 X SSPE, 1.0 % (w/v) SDS	65 <sup>0</sup> C, 30 min X 2 washes
Wash II	2 X SSPE, 0.5% (w/v) SDS	65 <sup>0</sup> C 30 min X 2 washes.
Wash III	2 X SSPE	25 <sup>0</sup> C 30 min X 2 washes.

After the 2 X SSPE washes, the level of the background radioactivity attached to the membrane was assessed using a Geiger counter. If a higher degree of stringency was required, then the above solution was diluted appropriately. After washing, the membrane was partially air-dried, wrapped in Saran wrap and exposed to X-ray film for 12-96 h at -70°C.

To reprobe the membrane, the DNA probe was removed by incubating the membrane in 5 mM Tris-Cl pH 8.0, 2 mM EDTA, 0.1 X Denhart's solution for 1 h at 65°C. This was generally sufficient to remove all bound probe. Removal of the probes was monitored using the geiger counter and the membrane judged to be successfully stripped when radioactive counts were no longer detectable.

#### **2.3.4. DNA sequencing**

##### **2.3.4.1. Preparation of double stranded sequencing template**

Plasmid DNA was routinely used as double-stranded sequencing template. DNA was isolated by alkaline lysis as described in section 2.3.1.1 RNA was removed by treating the sample with 1/10th volume of 0.5 mg/ml RNase at 37°C for 1 h. The DNA was further purified by use of GeneClean glass bead solution ('glass milk') (Biolab 101 inc.) according to the manufacturer's protocol as described in section 2.3.5.2.. 10 µg of plasmid DNA was diluted to a final volume of 32 µl and denatured for 10 min at room temperature following the addition of 8

μl of 2 M NaOH. The NaOH was neutralised by the addition of 7 μl of 3 M sodium acetate pH 4.8 and 4 μl of distilled water. DNA was then precipitated upon addition of 3 volumes of ethanol and incubation at -80°C for 10 min. After centrifugation, the pellet was washed in 70% (v/v) ethanol, air-dried and resuspended in 14 μl of distilled water.

Annealing of the primer to the template was achieved by addition to the resuspended template of 2 μl of 10 mM primer and 2 μl of annealing primer. The tube was vortexed gently and incubated at 65°C for 5 min. The tube was then transferred to 37°C and incubated for 10 min. The tube was then placed at room temperature for 5 min. and then centrifuged briefly. After this step, the sequencing reaction proceeded immediately.

#### **2.3.4.2. Sequencing reaction and sequencing gel**

Sequencing of the DNA fragment of interest was carried out according to the dideoxy chain terminator method of Sanger *et al.* (1977). Depending on the nucleotide sequence under analysis the DNA was primed with either reverse primer (5'-GGAAACAGCTATGACCATG-3') or GUS sequencing primer (synthetic oligonucleotide specific to the gene encoding β-glucuronidase) (5'-TCACGGGTTGGGGTTTCTAC-3').

Labelling and termination reactions of the DNA template were carried out according to the T<sup>7</sup> sequencing<sup>TM</sup> kit protocol (Pharmacia). Labelling of the DNA was by incorporation of α-[<sup>32</sup>P] dATP during the second strand synthesis. The DNA was heated at 80°C for 2 min and fractionated by electrophoresis on a 6% (w/v) acrylamide, 7 M Urea, 1 X TBE (0.09 M Tris-Borate, 2 mM EDTA, pH 8.0) gel. Prior to loading samples, the gel was pre-run at 2,500 V until a gel

temperature of 50°C was attained. The running buffer used was 1 X TBE and gels were routinely run at a voltage sufficient to maintain a constant temperature of 50°C throughout the gel.

Subsequently, the gel was fixed for 30 min in 10% (v/v) acetic acid and 10 % (v/v) methanol and transferred to a piece of Whatman 3 MM filter paper. This was then dried down at 80°C for 1-2 h using a Bio-Rad gel drier, having first covered the gel in a sheet of cling film.

### **2.3.5. DNA manipulation and modification**

#### **2.3.5.1 Vector preparation for the receipt of DNA insert**

The vector used for insertion of DNA fragment of interest was digested with the appropriate restriction endonuclease(s) as described in section 2.3.1.5. Removal of the enzyme after digestion was achieved by addition of an equal volume of phenol:chloroform: isoamyl-alcohol (25:24:1 by volume). The sample was vortexed for 5 sec and centrifuged at 10,000 rpm for 30 sec. The resulting supernatant was transferred to a fresh tube and DNA precipitated by the addition of 0.1 volume sodium acetate (pH 4.8) and 2 volumes ethanol, and centrifugation at 12,000 rpm for 15 min at 4°C. The DNA was resuspended in sterile distilled water and the concentration estimated by electrophoresis of an aliquot through 0.8% (w/v) agarose.

#### **2.3.5.2. Purification of DNA fragments from agarose gels**

Following restriction digestion of the appropriate plasmid, the fragment of interest was separated from other DNA molecules by electrophoresis through a

0.8% (w/v) agarose gel and was purified from the agarose gel using the GeneClean kit (BIO 101 Inc.). All the required solutions were provided by the manufacturers.

The desired band was excised from an ethidium bromide stained gel and the gel slice placed in a 1.5 ml microcentrifuge tube. Having determined the weight of the gel slice, three volumes of NaI solution (90.8 g NaI, 1.5 g Na<sub>2</sub>SO<sub>3</sub> per 100 ml ) were added and the gel slice incubated at 50°C until completely melted. To this was then added 10 µl of glassmilk, an additional 1 µl of glass milk was added for each 0.5 µg of DNA above 5 µg in the gel slice. The tube was mixed well and incubated on ice for approximately 30 min to ensure adequate binding of DNA to the silica matrix. The glass beads were pelleted by centrifuging the tube for 5 sec at top speed in a microcentrifuge and NaI supernatant discarded. The pellet was resuspended in 400 µl of ice-cold NEW WASH (50% (v/v) ethanol, 0.1 mM NaCl, 10 mM Tris-Cl pH 5.7, 1 mM EDTA pH8.0) and spun for 5 sec as before. Discarding the supernatant, the wash procedure was repeated twice more. After the supernatant from the third wash was removed, the tube was spun again for a few seconds and all remaining traces of liquid carefully removed. In order to dilute the DNA from glassmilk, the pellet was resuspended in 10 µl of distilled water and incubated at 55°C for 3 min. After centrifuging for 30 sec, the supernatant containing the diluted DNA was carefully transferred to a fresh tube. The elution procedure was repeated once more to ensure that all the DNA had been recovered from the silica and this second eluate added to the first. An aliquot was electrophoresed on a TAE agarose gel in order to estimate quantity and quality of the recovered DNA. The remaining aliquot was stored at - 20°C until required.

#### **2.3.5.3. End filling of restriction enzyme-generated 5' overhangs**

The cloning of fragments containing 5' overhangs resulting from restriction digestion, into blunt-ended sites within the chosen vector, necessitated the end-filling of these overhangs. This was achieved by the addition of 1/10th volume of 2 mM of all 4 dNTP's, 1/10th Nick Translation buffer (50 mM Tris-HCl pH 7.5, 10 mM Magnesium Sulphate, 0.1 mM DTT, 50 µg/ml BSA) and 10 units Klenow DNA polymerase to the restriction mix immediately following digestion. The mixture was then incubated at room temperature for approximately 30 min. The reaction was stopped by adding EDTA to a final concentration of 25 mM and DNA was recovered with phenol-chloroform extraction and ethanol precipitation.

#### **2.3.5.4. Removal of 5' terminal phosphates from restriction endonuclease treated DNA**

The probability that a single-cut vector would re-ligate due to the presence of compatible ends was significantly reduced by the removal of the phosphate groups present on the 5' termini of the cut DNA. This was achieved by the use of calf intestinal alkaline phosphatase (CIP). Following digestion of the vector with appropriate restriction enzyme, the digest mix was phenol-extracted, ethanol precipitated, dried and resuspended in a small volume of sterile distilled water. 10 X CIP buffer (100 mM, Tris-Cl pH 8.5, 10 mM Mg Cl<sub>2</sub>, 10 mM ZnCl<sub>2</sub>) was added to a 1X final concentration. One unit of CIP enzyme was added and the mix incubated for 30 min at 37°C. The dephosphorylated vector was then re-extracted with phenol-chloroform, ethanol precipitated, dried and resuspended in sterile distilled water.

### 2.3.5.5. Ligation of the prepared insert and vector

100 ng of digested, dephosphorylated vector was mixed with a sufficient concentration of insert to ensure a three-fold excess of insert ends. The concentration of ends was determined as follows

$$(2 \times 10^6) / (660 \times \text{number of bases}) = \text{pmol ends} / \mu\text{g double stranded linear DNA}.$$

To the vector plus insert was added 1/5th final volume of DNA ligase buffer (0.25 M Tris-Cl pH 7.6, 50 mM MgCl<sub>2</sub>, 50 mM DTT, 250 ug/ml BSA ) and 1-10 units of T4 DNA ligase. Sterile distilled water was added to make a final volume of 10 -50  $\mu$ l and the mix incubated either 37°C for 1 h or overnight at 16°C. Blunt-ended ligation was carried out essentially as above, but with the addition of 1/10 th volume of 10 mM hexamine cobalt chloride to the reaction before adding the ligase. 1-2 min following addition of the enzymes, 1/5th volume of 100 mM NaCl was added. These conditions were found to greatly enhance the ligation efficiency in the blunt-ended ligation reactions.

### 2.3.5.6. Preparation *E.coli* competent cells

A single colony of the *E. coli* strain XL1-Blue was inoculated into 5 ml of LB containing tetracycline at a concentration of 12.5  $\mu$ g/ml and grown at 37°C with shaking at 250 rpm overnight to stationary phase. The following day, an aliquot of this culture was diluted 1:250 with LB and incubated at 37°C with constant agitation for 3-4 h until an OD<sub>600</sub> of 3.5-5.5 was attained. Bacteria were recovered by centrifugation in a small sorvall RC-5B centrifuge at 4,000 rpm for 10 min at 4°C having first pre-cooled the rotor. The resultant pellet was gently resuspended

in a 1/2 volume of ice cold 50 mM CaCl<sub>2</sub>, and incubated on ice for 30 min. Following this, cells were spun down as before, resuspended in 1/10th volume ice-cooled 50 mM CaCl<sub>2</sub>, 20% (v/v) glycerol and dispensed into 100 µl aliquots. Cells were flash-frozen in liquid nitrogen and stored at -80°C.

#### **2.3.5.7. Transformation of *E. coli* with plasmid DNA**

Approximately 10-100 ng plasmid was added to 100 µl of transformation competent *E. coli* XL1-Blue and incubated on ice for 30 min. Cells were then heat-shocked at 40°C for 2 min. 1 ml of LB, pre-warmed to 37°C, was added and the cells incubated at 37°C for 1 h. Following recovery of the cells by centrifugation for 1 min, cells were gently resuspended in 100 µl LB and spread on LB containing the appropriate antibiotic for selection of transformed cells. These were then incubated at 37°C overnight and potential recombinants investigated by restriction digest.

#### **2.3.5.8. Screening of transformed bacterial colonies by α-complementation**

Where selection on a colorigenic basis was appropriate, 50 µl 250 mg/ml 5-bromo-4-chloro-β-D-galactoside (X-gal) and 50 µl 0.5 M isopropyl-β-D-thiogalactopyranoside (IPTG) was added to each plate by spreading on the surface prior to the application of the cell suspension. Plates were then incubated at 37°C overnight. Bacteria transformed with non-recombinant vector form blue colonies in the presence of X-gal on derepression of the lacZ gene fragments by IPTG. Insertion of the recombinant fragment into the cloning site of the vector however disrupts the lacZ N-terminal fragment and colonies containing recombinants plasmids are white.



## **2.4. Production and analysis of transgenic cucumber hairy roots**

### **2.4.1. Construction of promoter $\beta$ -glucuronidase gene fusion for plant transformation**

Putative promoter fragments to be used were subcloned into the plant transformation vector pBIN19 and used to transform *E.coli* as described in section 2.3.5.7. These recombinant vectors were then introduced into *Agrobacterium rhizogenes* using a freeze thaw direct transformation procedure. Recombinant plasmid carrying the desired fragment was analysed by restriction analysis of minipreparation of plasmid DNA.

### **2.4.2. Transfer of recombinant plasmid pBIN19 into *Agrobacterium rhizogenes* by freeze thaw direct transformation**

A single colony of the *Agrobacterium rhizogenes* strain A4 was inoculated into 5 ml LB containing 100  $\mu$ g/ml rifampicin and 500  $\mu$ g/ml streptomycin and grown at 30°C for 24-48 h to stationary phase. The culture was then diluted 1:100 in LB and incubated at 30°C for a few hours until OD<sub>600</sub> of 0.5-0.6 was attained. The bacteria were recovered by centrifugation at 4,000 rpm for 10 min at 4°C having first pre-cooled the rotor. The resultant pellet was gently resuspended in 2 ml of ice-cold 50 mM CaCl<sub>2</sub> and dispensed into 200  $\mu$ l aliquots. Cells can be directly used for transformation or flash frozen in liquid nitrogen and stored at -80°C.

Approximately 1  $\mu$ g of recombinant plasmid DNA was added to 200  $\mu$ l of *Agrobacterium* competent cells. The tubes were mixed and flash frozen in liquid nitrogen immediately. The tubes were then thawed by incubating at 37°C for 5

min. 1 ml of LB containing 5 mM glucose was added, mixed and the tubes incubated at 30°C for at 2-3 h with gentle shaking. After centrifugation for 30 sec, the supernatant was discarded and the cells were resuspended in 100 µl LB and spread on LB plates containing 100 µg/ml kanamycin, 100 µg/ml rifampicin and 500 µg/ml streptomycin for selection of transformed cells.

### **2.4.3. Cucumber hypocotyl transformation and regeneration of hairy roots**

#### **2.4.3.1. General conditions**

Cultivation of all genetically manipulated plants and bacteria was carried out according to the code of practice of the local genetic manipulation safety committee. All the tissue culture work operations were carried out in a laminar flow cabinet using standard aseptic technique. All plants in culture were kept in growth room with 24 h illumination from white fluorescent tubes (Thorn 3500) at a constant temperature of 25°C.

#### **2.4.3.2. Preparation of bacterial suspension for inoculation of hypocotyls**

A single colony of *A. rhizogenes* strain A4 carrying the recombinant pBIN19 of interest was used to inoculate 5 ml of LB medium containing 50 µg/ml kanamycin, 100 µg/ml rifampicin and 500 µg/ml streptomycin. The bacteria were grown with shaking at 300 rpm for 24 to 36 h at 30°C. This culture was then harvested by centrifugation and resuspended in 5 ml MSO (basal medium of Murashige and Skoog without any hormone) liquid medium (Sigma).

#### 2.4.3.3. Preparation of cucumber hypocotyls for transformation

*Cucumis sativus* seeds (cv Marketmore) were soaked in 70% (v/v) ethanol for 1 min and 20 min in 10% (v/v) chlorox (commercial bleach). Seeds were then rinsed several times with sterile double distilled water followed by soaking in 25 µg/ml rifampicin for 2 h. Seeds were then placed onto MS medium containing 1% (w/v) sucrose, 1% (v/v) of 5% (w/v) MES pH 5.7, 0.8 % (w/v) agar, and pH adjusted to 5.7 with 2.0 M NaOH for 3 days in the dark and two days in the light. Hypocotyls were excised (approximately 1-2 cm) for transformation and placed in 30 ml of MSO liquid medium containing 20 µM acetosyringone (Aldrich).

#### 2.4.3.4. Inoculation of hypocotyls and regeneration of transformed hairy roots

*A. rhizogenes* suspension (3 ml) was added to the hypocotyls in 30 ml MSO medium and left at room temperature for 1-2 h with gentle shaking (60 rpm). Hypocotyls were blotted on sterile filter paper and placed on MS agar (pH 5.7) plates (containing 200 mg/L  $\text{KH}_2\text{PO}_4$ , 2 mg/L ascorbic acid, 1 mg/L kinetin and 0.1 mg/L 2,4-dichlorophenoxyacetic acid) for 4 days co-cultivation in continuous light at 25 °C. The hypocotyls were then washed in MSO liquid medium containing 1 mg/ml vancomycin for 4 h with gentle shaking to kill the *Agrobacterium*. After blotting to remove excess liquid, the hypocotyls were placed on MS agar plates (containing  $\text{KH}_2\text{PO}_4$ , ascorbic acid, kinetin and 2,4-dichlorophenoxyacetic acid with concentrations as above pH 5.7) containing 500 µg/ml vancomycin. Cucumber hypocotyl segments were incubated horizontally. Roots appearing within two weeks from non-wounded sites were assumed to be non-transformed and were removed. Hairy roots formed after two weeks, from the callus at the cut ends, were recognised by their size and appearance. These roots were then transferred onto the same medium but with the presence of 50 µg/ml

kanamycin. Hairy roots were subcultured every 2 weeks by removing the terminal 1-2 cm onto fresh medium. Only transformed hairy roots grew in the presence of kanamycin.

## **2.5. Production of transformed plants**

### **2.5.1. Regeneration of transformed *Nicotiana plumbaginifolia***

Tobacco leaf disc transformation was essentially as described in Draper *et al.* (1988). Tobacco leaves were washed briefly in 70% (v/v) ethanol and surface-sterilised in a 10% (v/v) solution of sodium hypochlorite for 15 min. The leaves were rinsed several times in large amount of sterile distilled water. Leaf explants were dissected from the leaf lamina taking care to avoid the mid-rib. The explants were then incubated in a 1/20 solution of the desired recombinant *Agrobacterium tumefaciens* in shooting medium (refer section 2.2.4). Inoculated explants were then transferred onto shooting medium plates containing 0.8% (w/v) agar and incubated at 25°C for two days. After this period of time, the explants were then transferred onto shooting medium plates containing 500 µg/ml carbenicillin, 200 µg/ml kanamycin and culturing continued under the same conditions to select for transformed cells.

Following a period of a few weeks in culture, explants begin to produce shoots as a result of the regenerative capacity of transformed cells on kanamycin containing medium. Such shoots were aseptically excised from the explants taking care to avoid taking any callus material and placed onto expansion medium (refer section 2.2.4) containing 250 µg/ml carbenicillin and 100 µg/ml kanamycin. This facilitates rooting of shoots while maintaining selection. Shootlets which developed and rooted, were transferred to Levington Universal potting compost

and incubated at 25°C in a 16 h photoperiod at an irradiance of 100  $\mu\text{mol m}^{-2}\text{s}^{-1}$ . The shootlets in pots were first covered with plastic bags which were gradually pierced to allow acclimatisation to the growth room environment and eventually removed after approximately 2 weeks. Growth was continued until the plants produced seeds. *N. plumbaginifolia* plants were allowed to self-fertilise and seeds were collected from each plant and stored at room temperature.

### **2.5.2. Germination of transgenic *N. plumbaginifolia* seeds**

Seeds were surface sterilised by treatment with 10% (w/v) sodium hypochlorite for 10 min after which time the solution was removed and the seeds rinsed 6 times with sterile double distilled water. Seeds were imbibed in 1 mM gibberellic acid overnight at 4°C and rinsed the following day in sterile double distilled water. The seeds were then transferred to moist filter paper without antibiotic. The plates were then incubated in a 16 h photoperiod at 25°C.

### **2.5.3. Selection of germinating seeds at specific developmental stages**

Since germination of *N. plumbaginifolia* is not synchronous, it is necessary to select seedlings of specific stages for analysis. Seedlings at stage 0, (seed immediately following imbibition) stage 4, (radicle is approximately twice the length of the seed coat) and stage 7, (green seedlings) were selected using a binocular microscope and stored at -70°C.

## **2.6 Expression analysis of transgenic tissues**

### **2.6.1. Northern analysis of hairy roots**

Hairy roots were incubated in the dark (wrapped in aluminium foil) in a petri dish containing filter paper soaked in sterile double distilled water or 25 mM sucrose. After 2, 4 and 6 days, hairy roots from each treatment were transferred to liquid nitrogen and kept at - 70°C. The RNA was isolated and analysed by northern blotting.

### **2.6.2. Fluorometric assay of $\beta$ -glucuronidase**

GUS enzyme activity was determined according to Jefferson (1987) by measuring the fluorescence of methylumbelliferone produced by GUS cleavage of methylumbelliferyl- $\beta$ -D-glucuronide (MUG).

Approximately 0.2 g of plant material, depending on the tissue type being analysed, was homogenised in 400  $\mu$ l of GUS extraction buffer (GEB) (50 mM Sodium Phosphate buffer pH 7.0, 10 mM EDTA, 0.1% (v/v) Triton X-100, 0.1% (w/v) sarkosyl, 10 mM  $\beta$ -mercaptoethanol.) and clarified by centrifugation at 13,000 rpm for 10 min. 100  $\mu$ l of the cleared extract was used per GUS enzyme assay. The remaining extract was frozen in liquid nitrogen and later used for protein concentration estimation.

Reactions were initiated by the addition of 100  $\mu$ l of the extract to 900  $\mu$ l of 5mM MUG in GEB. Immediately after the addition and mixing of sample, 100  $\mu$ l aliquot was removed and added to 900  $\mu$ l of STOP (0.2 M Na<sub>2</sub>CO<sub>3</sub>, pH 11.2) and the remaining reaction mix incubated at 37°C for 60 min. The concentration of

MU (4-methylumbelliferone), the product of this reaction, was determined by measuring its fluorescence using a Perkin-Elmer LS series spectrofluorimeter with excitation at 365 and emission at 455 nm. The spectrofluorimeter was calibrated before each use with known standards of MU such that 600 nM MU was equivalent to 600 units of relative fluorescence. The relative fluorescence of the samples could therefore be read directly as nM MU. The rate of reaction for each sample can therefore be calculated from the change in MU concentration with time.

For the fluorogenic assay of GUS activity in *N. plumbaginifolia* seedlings, 100 seeds from each stage were homogenised in a total volume of 100  $\mu$ l GUS extraction buffer. The homogenate was centrifuged and 50  $\mu$ l of the cleared extract was used for enzyme assay as describe above.

### **2.6.3. Histochemical assay of $\beta$ - glucuronidase**

GUS histochemical analysis of transgenic hairy roots was carried out using a modified version of the method described by Jefferson (1987). Treated hairy roots were incubated in 1 mg/ml X-gluc (5-bromo-4-chloro-3-indolyglucuronide). X-gluc solution was prepared by dissolving the solid in dimethylformamide (DMF) to a concentration of 100 mg/ml with subsequent dilution to 1 mg/ml in 50 mM sodium phosphate pH 7.0 ) at 30<sup>0</sup>C for the appropriate length of time. The length of time required for a blue colour to appear was dependent on the level of GUS in the transgenic material. Generally, staining was monitored regularly for up to 4 h, after which staining was left to progress overnight. Material thus stained was cleaned by transferring to ethanol and incubating at room temperature overnight. Samples were stored in ethanol indefinitely. In stage 4 seedlings, prior

to incubation, fine forceps were used to remove the embryo from the seed coat and endosperm.

## **2.7. Sugar content estimation**

Sugars were extracted as described by McLaughlin and Smith (1994) and assayed with a Boehringer Mannheim sucrose analysis kit. Because of the small amounts assayed, samples were first treated with invertase before assaying glucose and glucose 6-phosphate. Results therefore represent the combined amount of glucose, from sucrose, free glucose and glucose 6-phosphate.

## **2.8. Protein concentration estimation**

Protein estimation was carried out essentially as described by Bradford (1976).



# **CHAPTER THREE**

## **RESULTS AND DISCUSSION**

# CHAPTER THREE

## MALATE SYNTHASE AND ISOCITRATE LYASE

### GENE EXPRESSION IN CUCUMBER ROOTS

#### 3.1. Rationale

The role of the glyoxylate cycle for conversion of storage lipids into sugar during germination and seedling development has long been established following the discovery of two key enzymes, ICL and MS. These glyoxylate cycle enzymes have also been discovered at other stages of plant development including late embryogenesis (Comai *et al.*, 1989; Turley and Trelease, 1990), pollen development (Zhang *et al.*, 1994) and senescence of leaves, cotyledons and petals (Pistelli *et al.*, 1991; Graham *et al.*, 1992; McLaughlin and Smith 1995).

Glyoxylate cycle enzymes are also synthesised in cells and tissues in response to sugar deprivation. The function of the glyoxylate cycle in such cells is unknown, but an anaplerotic rather than gluconeogenic function has been suggested (Graham *et.al.*, 1994a; Kim and Smith, 1994a). The control of gene expression during seed germination and during sugar deprivation is mediated by distinct *cis*-acting DNA sequences (Sarah *et al.*, 1996), indicating regulation of gene expression in response to different signals, possibly reflecting different functions for the glyoxylate cycle. *Ms* and *Icl* gene expression has been widely studied in order to analyse plant gene expression as has been discussed in Chapter 1. *Ms* and *Icl* are coordinately regulated in a variety of tissues and are subject to metabolic control. Throughout plant development, the expression of *Ms* and *Icl* is observed as early as in post germinative growth of oilseeds, but then declines when the plant reaches maturity. However reactivation of the genes takes place when the plant senesces.

Regulation of *Ms* and *Icl* gene expression by sugar metabolism has so far been demonstrated in cultured cells (Graham *et al.*, 1994a), protoplasts (McLaughlin and Smith, 1994; Graham *et al.*, 1994b; Reynolds and Smith, 1995b) and detached organs (Graham *et al.*, 1992; McLaughlin and Smith 1994). It has also been demonstrated in whole seedlings and plants placed into darkness for prolonged periods (Graham *et al.*, 1992; Reynolds and Smith, 1995b; Sarah *et al.*, 1996). However in no case has it been shown that carbohydrate deprivation can lead to an activation of glyoxylate cycle enzyme synthesis in plants growing under natural conditions. In order to investigate this matter, roots (a sink organ) were chosen, in which sugars may affect metabolism and gene expression (Brouquisse *et al.*, 1991; Chevalier *et al.*, 1995; Williams and Farrar, 1990). Previously, *Ms* gene expression was detected in detached cucumber roots (Graham *et.al.*, 1992), but the effect of sugar on *Ms* regulation has not been investigated. Furthermore, expression of the *Icl* gene was not investigated, and studies with starved maize root tips could detect MS but not ICL activity (Dieuaide *et.al.*, 1992). To further elaborate and define the expression of these genes in roots, the first aim of this part of the work was to determine if *Icl* and *Ms* gene expression is activated by sugar deprivation of detached cucumber roots. The second aim was to investigate regulation in roots of whole plants in which photosynthate supply to the roots was impaired by treatments expected to occur commonly under natural conditions.

### 3.2. Sugar effect on transcription of the Malate Synthase and Isocitrate Lyase genes in cucumber wild type roots

Previous results had shown that the *Ms* gene is expressed in detached cucumber roots (Graham *et al.*, 1992), but the effect of sugar in regulating *Ms* gene expression is lacking. Furthermore, the expression of *Icl* which is coordinately regulated with *Ms* (Comai *et al.*, 1989) was not investigated. In order to investigate the regulation by sugar of *Icl* and *Ms* gene expression in detached wild type roots, cucumber (*Cucumis sativus* L. cv Marketmore) seeds were germinated on filter papers for five days in a 16 h photoperiod at 25°C. Thereafter, the roots (terminal 1 cm) were detached from the seedlings after five days growth and incubated in the dark on filter paper in the presence of water or 25 mM sucrose for 2 day intervals up to 6 days (Figure 3.1). About 100 roots were used for each treatment. Total RNA was isolated and 10 µg aliquots of RNA samples were run on the gel with 3 day cotyledon RNA which served as a marker. Northern hybridization analysis was carried out employing the 1.4 kb insert from pBSICL and the 1.9 kb insert from pBSMS 1.9 as probes. The filter was first hybridised with the *Icl* cDNA probe, radiolabelled with [<sup>32</sup>P]dCTP and subsequently, following removal of the first probe, rehybridised with the *Ms* cDNA probe similarly labelled with [<sup>32</sup>P]dCTP. The results are displayed in Figure 3.2.

The results show that the *Icl* transcript is approximately 2.1 kb long which agrees with the predicted size from data gained from analysis of the genomic clone (Reynolds and Smith, 1995a), whereas the *Ms* transcript is about 1.9 kb (Graham *et al.*, 1989). The two genes are transcribed in a coordinate manner, which agrees with previous observations that the level of translatable mRNA and enzyme activities of the two genes are coordinately regulated (Becker *et al.* 1978; Weir *et al.*, 1980). The level of *Ms* and *Icl* expression increases dramatically at the later



Figure 3.1. a). 5 day old cucumber (*Cucumis sativus* L. cv Marketmore) seedlings.  
b) Incubation of cucumber root tips with or without sucrose in petri dish.

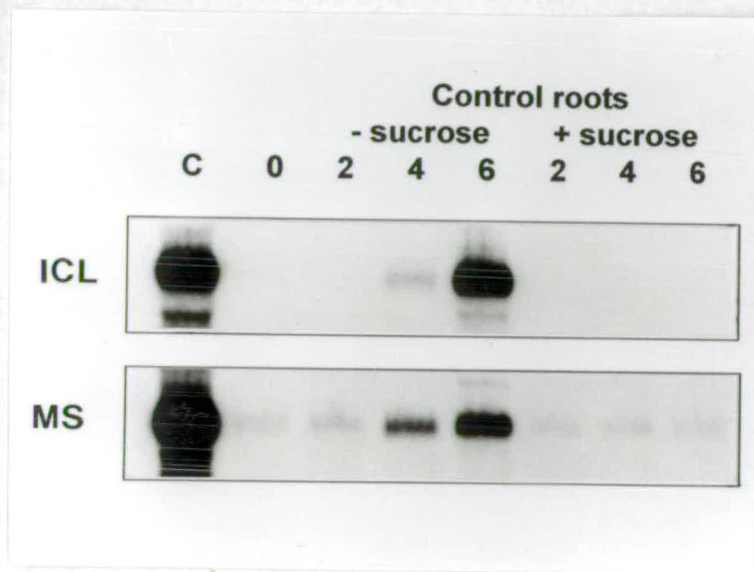


Figure 3.2. Effect of sucrose on expression of *Icl* and *Ms* genes in detached roots. The terminal 1 cm of roots from 5 day seedlings (control roots ) were detached and incubated in the absence or presence of 25 mM sucrose for 0, 2, 4 and 6 days as shown. RNA was then isolated and 10  $\mu$ g of each analysed by northern blotting using *Icl* and *Ms* cDNA probes. Lane C shows the hybridisation signal obtained with 10  $\mu$ g RNA from cotyledons of seeds 3 days post-imbibition



stages of the treatment when incubated in water. Furthermore the *Ms* transcript is detected as early as two days of incubation compared to *Icl* transcript which appears with a very low level at day four. On the contrary, in the presence of 25mM sucrose, both *Ms* and *Icl* genes are almost completely repressed throughout the six days incubation. The results demonstrate that both *Ms* and *Icl* genes are expressed in roots and are subject to sucrose regulation which agrees with previous studies that demonstrated such regulation in cultured cells and detached cotyledons (Graham *et al.*, 1994a; McLaughlin and Smith, 1994).

### 3.3. Establishment of hairy root cultures of cucumber

As has been previously mentioned, analysis of *Ms* and *Icl* gene expression employed the *Nicotiana* heterologous gene expression system and cucumber protoplast system (Sarah *et al.*, 1996; Reynolds and Smith, 1995b). It would be better if homologous gene expression could be employed in stable transformants but this is difficult as transgenic cucumber plants are difficult to produce. In order to resolve this matter, transgenic hairy roots were regenerated and subsequently used to study *Ms* and *Icl* homologous gene expression.

Hairy roots were generated by using *Agrobacterium rhizogenes* strain A4. Cucumber seed were germinated on M&S medium followed by incubation for three days in the dark and then two days in the light. Hypocotyls of the five day seedlings were excised and infected with *Agrobacterium rhizogenes*. After infection, the hypocotyls were transferred onto M&S plates supplemented with 1mg/ml vancomycin. After two weeks pre-formed roots (wild type) were produced from the wounded site and excised. The hypocotyls were transferred onto new M&S plates which contained 100 µg/ml kanamycin together with 1 mg/ml of vancomycin. After about three weeks to one month, the hairy roots started to grow

from the callus and this is clearly displayed in Figure 3.3. The efficiency of the cells at the wounded site being transformed is variable among the hypocotyls.

The roots induced by *Agrobacterium rhizogenes* can be distinguished morphologically and physiologically from normal roots as they have a faster growth rate, display bigger size and increased branching. One branch of hairy roots from a single hypocotyl, which was referred to as a single transformant, was transferred onto a different plate for propagation. After one month, a substantial amount of hairy roots were obtained from a single transformant increasing to many times its original size, with a massive net of roots formed from several new branches (Figure 3.4a). These hairy roots were then maintained by subculturing once a month on a plate containing a reduced concentration of vancomycin. *Agrobacterium rhizogenes* can induce hairy roots on many dicotyledonous plants, and so far 116 species from 26 families have been transformed (Tempe and Casse-Delbart, 1989; Tepfer, 1990).

### **3.4. Effect of sugar on Malate Synthase and Isocitrate Lyase genes in hairy roots of cucumber**

Previous results have shown that the expression of *Ms* and *Icl* genes has been detected in detached normal roots and is regulated by sugar. As hairy roots were now available from the inoculation of cucumber hypocotyls with *Agrobacterium rhizogenes* and subsequently maintained in axenic culture, a similar experiment as for normal roots of cucumber was carried in order to investigate the expression of both genes in hairy roots. For this purpose, a substantial amount of hairy roots from a single transformant was used.





Figure 3.3. Hairy roots emerging from callus at the wounded site of a cucumber hypocotyl after inoculation with *Agrobacterium rhizogenes* on MS medium and normal roots emerging from middle of the hypocotyl but not from the wounded site.

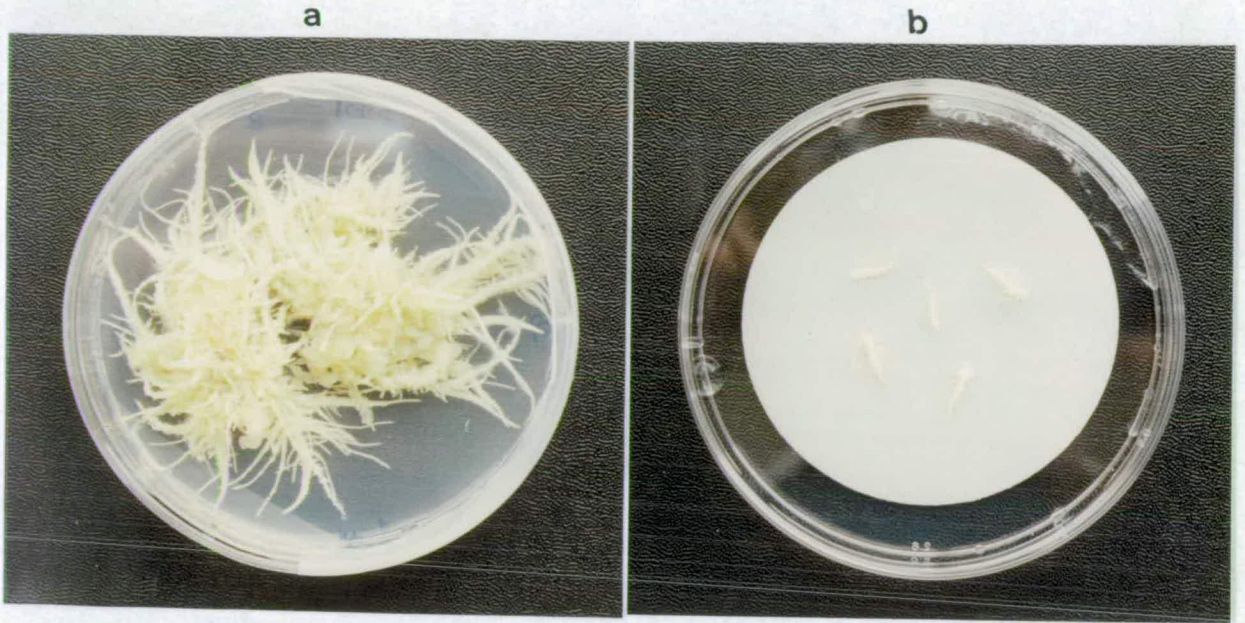


Figure 3.4. a) Appearance of hairy roots obtained from cucumber hypocotyls infected by *Agrobacterium rhizogenes* strain A4. This is one month old hairy roots after first subculturing. b) Photograph shows how the hairy roots are incubated with and without sucrose in the petri dish.



The 1 cm apices of hairy roots were excised and incubated as displayed in Figure 3.4b in the dark for two day intervals up to six days in the presence and absence of 25 mM sucrose. RNA was isolated from the hairy roots and was subjected to northern analysis using the 1.4 kb *Icl* cDNA as a probe. Following removal of *Icl* cDNA probe, the filter was then rehybridized with the 1.9 kb *Ms* cDNA probe. The result is presented in Figure 3.5. At day 0, both MS and ICL mRNAs are hardly detectable. Nevertheless, the amounts of both transcripts increase dramatically at day six in the absence of sucrose. On the contrary, when sucrose was added to the hairy roots which were kept in the dark up to six days, the induction of both MS and ICL mRNAs was inhibited completely. MS and *Icl* mRNAs seem to appear at a low level after 2 days starvation but then increased after 4 and 6 days starvation. The results presented here are essentially similar to the *Ms* and *Icl* expression in normal roots (Figure 3.2) suggesting that both genes are faithfully induced in hairy roots, leading to the hypothesis that such roots could be a good potential experimental system for genetic transformation studies to further analyse gene expression, since transgenic cucumber plants are difficult to produce. The activities of ICL and MS enzymes were assayed in extracts of both normal and hairy roots, and although very low, were shown to increase upon carbohydrate deprivation and were repressed by sucrose ( data of Dr. L. De Bellis, not shown).

### **3.5. Expression of *Ms* and *Icl* promoter GUS fusion in transgenic hairy roots**

In order to monitor gene expression and determine the regulatory properties of *Ms* and *Icl* promoters in response to sugar deprivation, transgenic hairy roots were created . The 2.9 kbp *Icl* promoter fused at the translational initiation codon to the GUS gene (Reynolds and Smith, 1995b) and in the other case, a 1076 bp MS

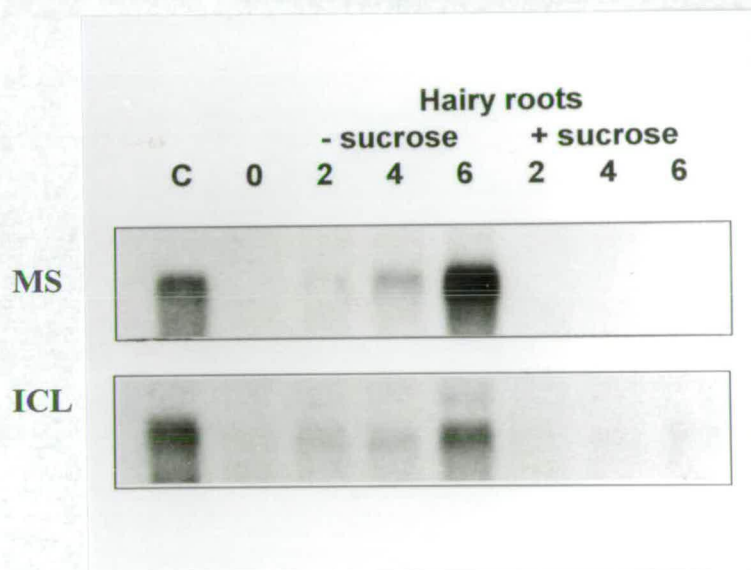


Figure 3.5. Effect of sucrose on expression of *Icl* and *Ms* genes in hairy roots.

1 cm sections of hairy roots from one month old axenic cultures were detached and incubated in the absence or presence of 25 mM sucrose for 0, 2, 4 and 6 days as shown. RNA was then isolated and 10  $\mu$ g of each analysed by northern blotting using *Icl* and *Ms* cDNA probes. Lane C shows the hybridisation signal obtained with 10  $\mu$ g RNA from cotyledons of seeds 3 days post-imbibition.



promoter fragment fused to GUS in a translational fusion (Graham *et al.*, 1990) were used (Figure 3.6). These constructs subsequently referred to as “full length promoters” were cloned into the plant transformation binary vector pBIN19 (Bevan, 1984). The resulting constructs were introduced into *Agrobacterium rhizogenes* using the freeze thaw direct transformation procedure.

Both constructs have previously been shown to respond normally to carbohydrate regulation in transgenic plants and electroporated cucumber protoplasts (Graham *et al.*, 1994b; Reynolds and Smith, 1995b; Sarah *et al.*, 1996). Multiple independent transgenic hairy roots were obtained for each construct by inoculation of cucumber hypocotyls. For GUS activity measurements, four different transformants of transgenic hairy roots were used, each of which was subjected to 2, 4 and 6 days incubation in the dark in the presence and absence of 25 mM sucrose. Extracts from each treatment were prepared and fluorometric assays were then carried out for each transformant. Results displayed in Figure 3.7a and 3.7b show the means of GUS activities in transgenic hairy roots of cucumber containing *Ms* and *Icl* promoter GUS fusions. Upon starvation of the hairy roots, the GUS activity for both *Ms* and *Icl* promoters increases to a detectable amount after 2 days and continued to increase during the next 4 days, whereas in the presence of sucrose, GUS activities were hardly detectable over the period of the incubation time. These patterns correspond to the distinctive features of expression of the genes in normal and hairy roots as determined by northern analysis (Figure 3.2 and 3.5).

These results have demonstrated that the *Ms* and *Icl* promoters are responsive to regulation of expression by sugar, indicating control of expression is primarily at the level of transcription. Nevertheless, the GUS activity from the *Ms* promoter was found to be about 4-fold higher than that of the *Icl* promoter. The

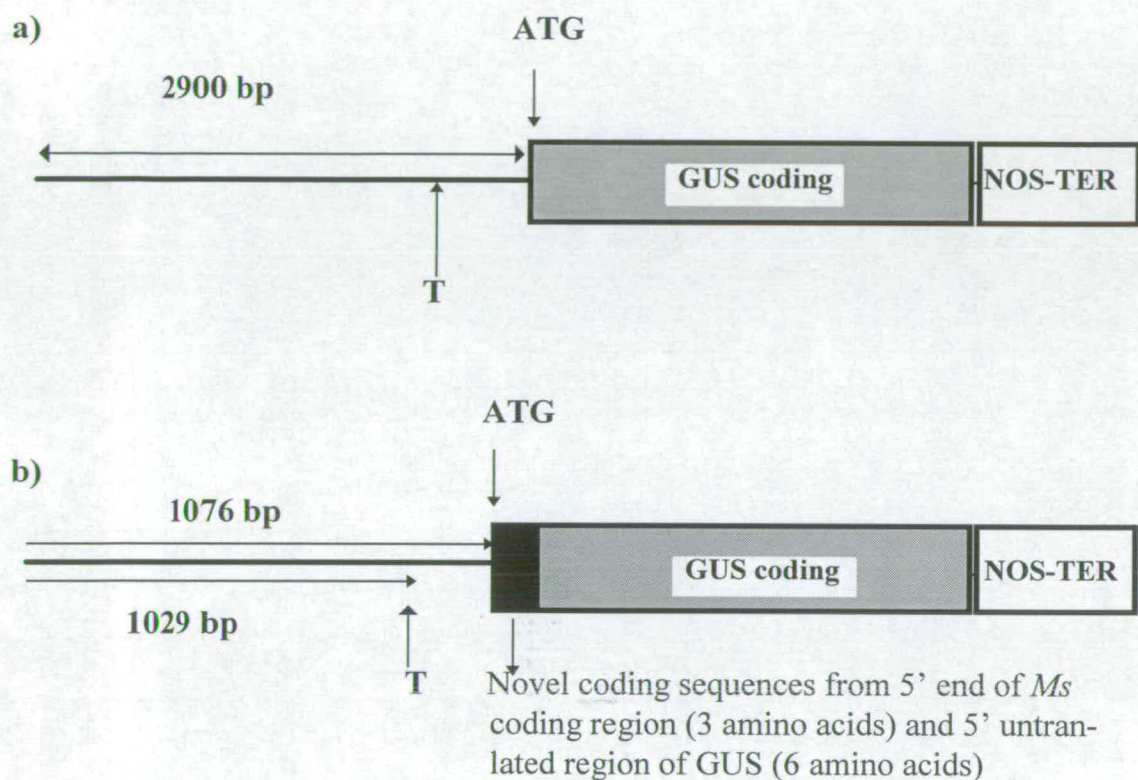


Figure 3.6 Schematic representation for *Icl* (Reynolds and Smith, 1995) and *Ms* (Graham *et al.*, 1990) promoter constructs. A) A 2.9 kbp *Icl* promoter fused at the translational initiation codon ATG to the GUS gene. B) A 1076 bp *Ms* promoter fragment fused to GUS in a translational fusion. The lower number shows 1029 bp upstream of the transcriptional initiation site. T represents the transcription start in each case.



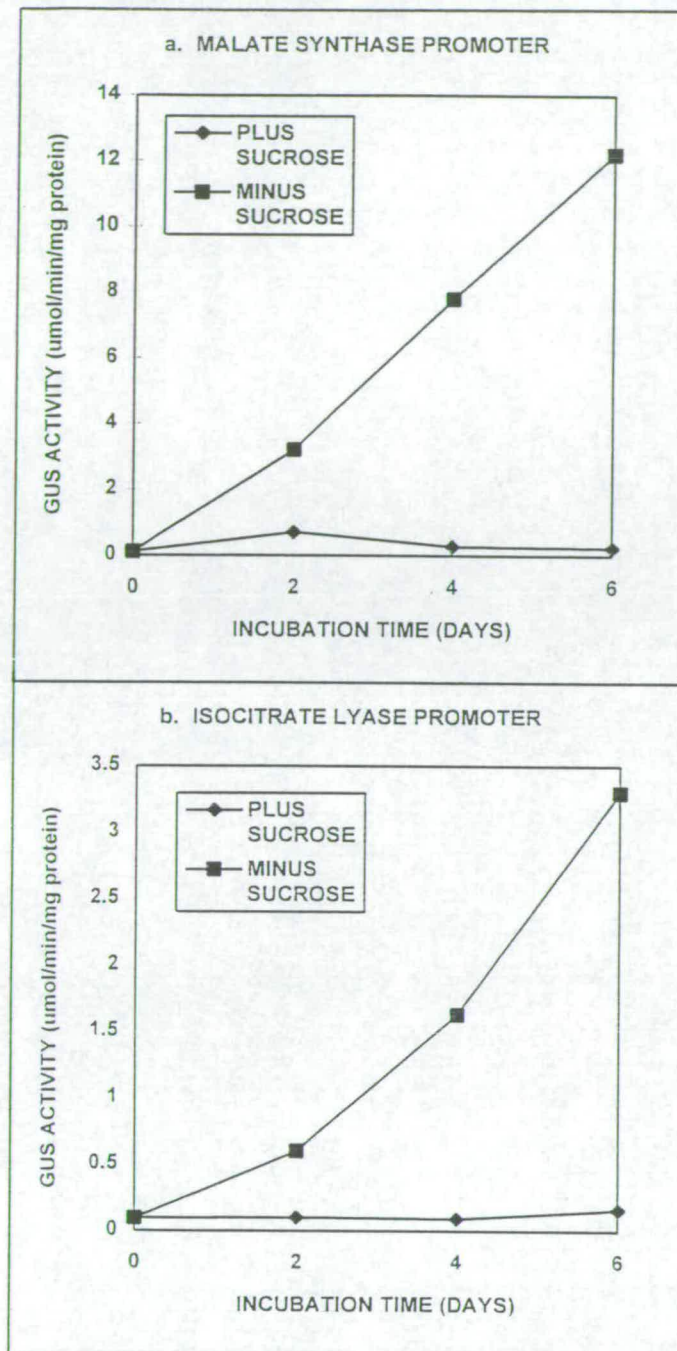


Figure 3.7. Effect of sucrose on GUS activity in transgenic hairy roots.

Transgenic hairy roots containing (a) *Ms*, and (b) *Icl* promoters fused to GUS were obtained. Sections (1cm) were detached and incubated in the absence and presence of 25 mM sucrose for up to 6 days as shown, then extracts prepared and GUS activities measured. Four independent transformants were assayed at each point, giving Standard Errors of less than 32% in all cases.

higher level of GUS activity from the *Ms* promoter may be explained by different translational fusions for each gene resulting in different GUS molecules with different activity or stability or that the *Ms* promoter is stronger than the *Icl* promoter under these conditions. The level of GUS activity is approximately two orders of magnitude greater than with the same genes in transgenic plants (Graham *et al.*, 1990; Sarah *et al.*, 1996; Reynolds and Smith, 1995b). Furthermore, the fold repression by sugar is also greater in hairy roots compared to those systems. These results suggest that transgenic cucumber roots are a superior system in which to analyse sugar regulation of expression of these genes.

### **3.6. Histochemical analysis of expression patterns of *Ms* and *Icl* promoter GUS fusions in detached transgenic hairy roots**

It has been demonstrated from GUS activities in figure 3.7 that the 5' flanking sequences of *Ms* and *Icl* genes faithfully direct the expression in transgenic roots. In order to investigate the spatial pattern of GUS activity driven by *Ms* and *Icl* promoters within the hairy roots, histochemical analysis of GUS activity was conducted. The terminal 1-2 cm of transgenic hairy roots containing the promoter GUS fusions were detached and incubated in the presence and absence of 25 mM sucrose for six days then stained histochemically for GUS activity. The staining patterns of hairy roots as a whole are shown in Figure 3.8. No GUS activity is apparent in non-transgenic hairy roots (data not shown). Transgenic hairy roots incubated in sucrose were stained very slightly but showed very strong GUS activity if they were deprived of sugar. In general, both *Ms* and *Icl* promoter GUS fusion constructs direct an equivalent staining of the whole transgenic hairy roots.



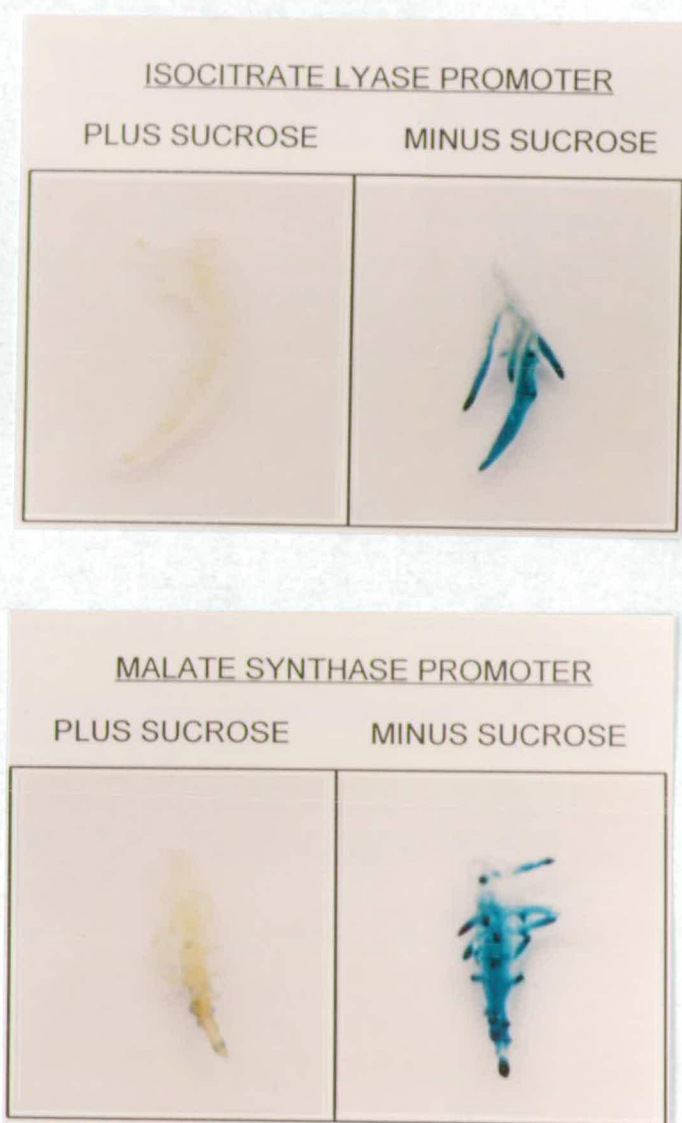


Figure 3.8. Effect of sucrose on histochemical staining of GUS activity in transgenic hairy roots.

Representative transgenic hairy roots were incubated in the presence or absence of 25 mM sucrose for 6 days then stained for GUS activity. Magnification x2

In Figure 3.9 the patterns of GUS expression in transgenic hairy roots containing both constructs, with magnification x10, are presented. In sugar-deprived roots, the GUS activities show a clear tissue specificity. For instance, in sugar-deprived roots both *Ms* and *Icl* promoters preferentially direct GUS expression in root tips and lateral root primordia ( Figure 3.9 a,b,d,e ) and in some cases in procambium and stele ( Figure 3.9 c,f ). The cells in which staining is observed are presumably those with a high metabolic activity and demand for respiratory substrates. When carbohydrate becomes limiting, glyoxylate cycle-enzymes may be synthesised to participate in metabolising substrates other than carbohydrates. The results which are shown here are those from a single transformant and essentially similar results were obtained from other transformants. Taken together, these data further support the use of hairy roots as a powerful system to further study the *Ms* and *Icl* sequences through which sugar controls the transcription of these genes. Furthermore, the hairy root is easy to produce and to maintain in axenic culture by subculturing. It could also easily be used to study different metabolites and inhibitors which regulate *Ms* and *Icl* gene expression by providing substrates and inhibitors in the culture medium.

### 3.7. Expression of *Ms* and *Icl* genes in roots of whole plants

Activation of *Ms* and *Icl* genes has been shown to be regulated by sugar in cultured cells (Graham *et al.*, 1994a), protoplasts (McLaughlin and Smith, 1994; Graham *et al.*, 1994b; Reynolds and Smith, 1995b) and detached organs (Graham *et al.*, 1992). Expression of both genes is also observed in whole seedling and in plants incubated in prolonged darkness (Graham *et al.*, 1992; Reynolds and Smith, 1995b; Sarah *et al.*, 1996). In addition to that, further studies of *Ms* and *Icl* genes in this project have demonstrated the regulation of their expression by sugar in roots,



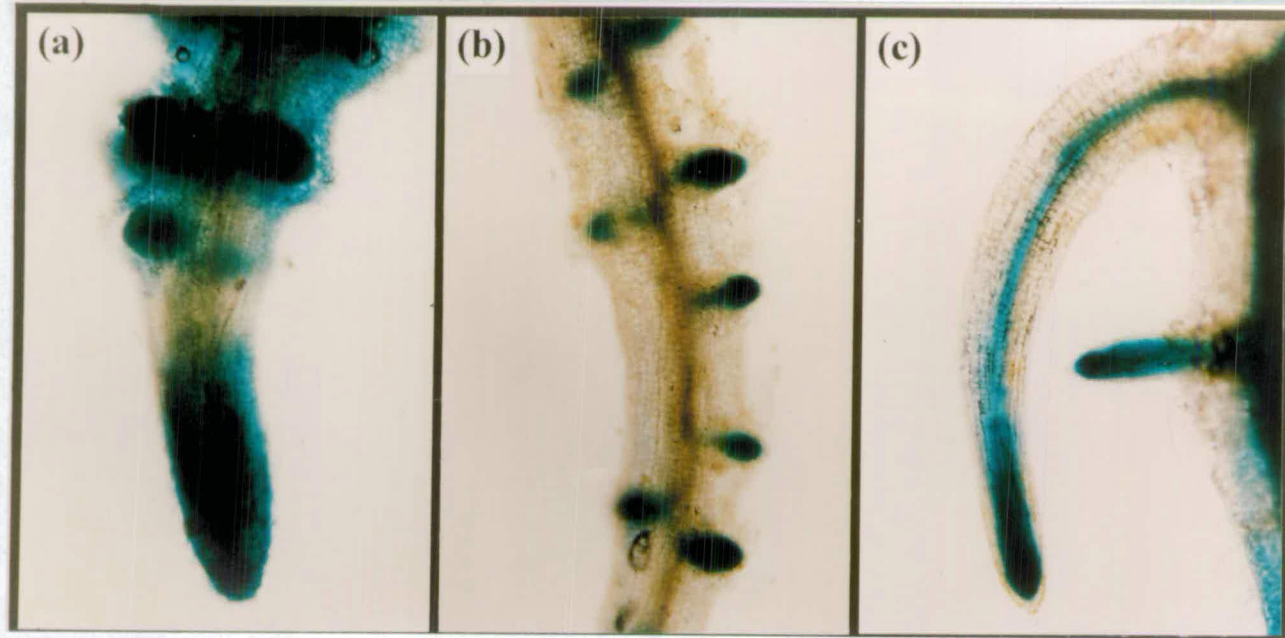


Figure 3.9. Histochemical localisation of GUS activity in sugar-deprived transgenic hairy roots.

Representative transgenic roots were incubated in the absence of sucrose for 6 days then stained for GUS activity. (a-c) Roots containing the *Icl* promoter fused to GUS reporter gene. (d-f) Roots containing the *Ms* promoter fused to GUS reporter gene. Magnification x10.

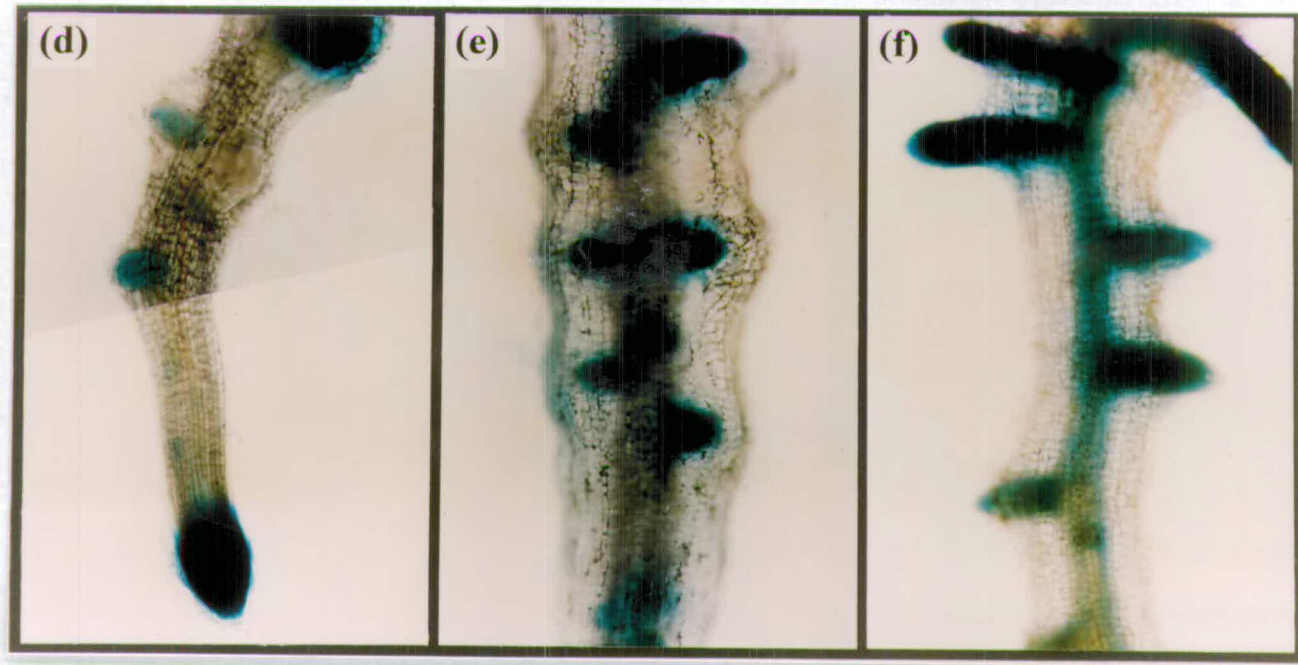


Figure 3.9



extending previous findings by Graham *et al.* (1992). The next experiment was set up in order to examine the expression of *Ms* and *Icl* genes in roots of whole plants under natural conditions in which carbohydrate supply to the roots becomes limiting. The hypothesis would be that such conditions may cause reduction of carbohydrate supply from leaves to roots, which subsequently leads to activation of *Ms* and *Icl* gene expression. The treatments imposed were shading and defoliation both of which mimic natural stress conditions of the plants. For this purpose, plants were grown and maintained in hydroponic medium with a relatively fast rate of growth. Under these conditions, roots could easily be harvested subsequently.

### 3.7.1. Shading Experiment

In this experiment, plants at the three-leaf stage were subjected to a 10 hours light / 14 hours dark cycle of different light intensities,  $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ ,  $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ ,  $2 \mu\text{mol m}^{-2} \text{s}^{-1}$  and total darkness for six days, a time period which was assumed to be sufficient to induce gene expression. Roots of three different plants from each treatment were harvested and combined together, RNA extracted and gel blot analysis carried out to examine *Icl* and *Ms* expression. As shown in Figure 3.10a the highest irradiance (  $200 \mu\text{mol m}^{-2} \text{s}^{-1}$  ) induced the lowest level of MS mRNA accumulation,  $20 \mu\text{mol m}^{-2} \text{s}^{-1}$  induced MS mRNA to a higher level, and  $2 \mu\text{mol m}^{-2} \text{s}^{-1}$  and total darkness showed the highest level of MS mRNA abundance. For *Icl* gene induction, a low amount of ICL mRNA could be detected under different light intensities but a significant amount was only seen under extreme shading or darkness. The glucose content of these roots was measured and the results are displayed in Figure 3.10b . A large amount of glucose was detected in the roots under  $200 \mu\text{mol m}^{-2} \text{s}^{-1}$  but the amount declined dramatically in response to shading and dark treatment. These results indicate that MS and ICL mRNA accumulation occurs under low light intensities in roots of

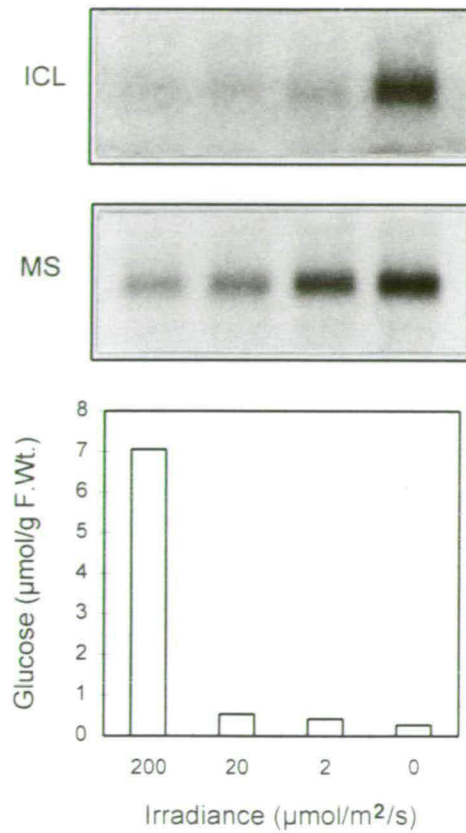


Figure 3.10. Effect of shading of cucumber plants on expression of *Icl* and *Ms* genes in roots.

a). Plants were grown to the 3-leaf stage then incubated for 6 days in different light intensities as shown. Total RNA was isolated from roots and 10  $\mu\text{g}$  of each analysed by northern blotting using *Icl* and *Ms* cDNA probes, as shown (upper panels). b) The sugar content of roots was estimated as total glucose ( free glucose plus glucose in sucrose ) (S. M. Smith, pers. comm.).



whole plants when carbohydrate supply is limiting. Further they establish a correlation between endogenous sugar content and *Icl* and *Ms* gene expression.

### 3.7.2. Defoliation experiment

For this experiment, plants at the three-leaf stage were subjected to varying degrees of defoliation, which might happen during grazing by animals or other mechanical damage. Defoliation treatments were (1) none, (2) both cotyledons, (3) both cotyledons and first leaf, (4) both cotyledons and first and second leaves. The third leaf was only about 1 cm in length, and therefore still a sink organ. The treated plants were then left for another six days. Similar to the shading experiment, the roots of the plants for each treatment were harvested, RNA extracted and ICL and MS mRNA abundance was assayed by northern blot analysis. The result displayed in Figure 3.11 shows that the induction of *Ms* and *Icl* genes is similar to that obtained with the shading experiment. In a complete plant, ICL mRNA is hardly detectable and it seems to be the same case in plants with both cotyledons removed and both cotyledons and first leaf removed. However, a substantial amount of ICL mRNA was detected in plants under severe defoliation where both cotyledons and first and second leaves were removed. For the *Ms* gene, the MS mRNA appears at a low level in a complete plant but upon defoliation the MS mRNA accumulated and was most apparent with the most extreme defoliation. The glucose content was measured in these roots and shown to decrease upon defoliation.

In both shading and defoliation experiments, the enzyme activities of MS and ICL were assayed in extract of roots (Dr. L. De Bellis, not shown). For the control plant, the enzyme activities were hardly detectable. Low levels of MS and

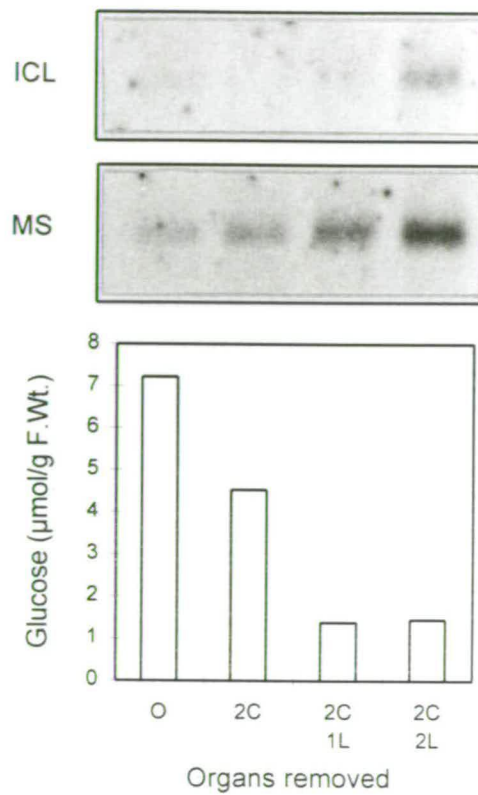


Figure 3.11. Effect of defoliation of cucumber plants on expression of *Icl* and *Ms* genes in roots.

A) Plants were grown to the 3-leaf stage then incubated for 6 days after removal of (O) no organs, (2C1L) both cotyledons plus first leaf, (2C2L) both cotyledons plus first two leaves. Total RNA was isolated from roots and 10 μg of each analysed by northern blotting using *Icl* and *Ms* cDNA probes, as shown ( upper panels ). B). The sugar content of roots was estimated as total glucose ( free glucose plus glucose in sucrose ) (S. M. Smith, pers. comm.).



ICL activities were detected in roots of plants subjected to the most extreme shading and defoliation experiments, although the activities from total homogenates of such roots was low and variable making it difficult to quantify reliably (data not shown). All the results gathered from both experiments are consistent with the hypothesis that gene expression is controlled by carbohydrate deprivation. It is proposed that the glyoxylate cycle serves to help maintain respiratory activity in roots when environmental factors limit carbohydrate supply from the shoot (see Chapter 6).

### 3.8. Discussion

#### 3.8.1. Regulation of *Icl* and *Ms* gene expression in roots by sugar

The results presented in this chapter have shown that *Ms* and *Icl* genes are expressed in response to carbohydrate deprivation of plant cells and tissues. Their expression in roots has elaborated and extended the previous results which showed *Ms* expression in detached cucumber roots. Furthermore the *Icl* expression and regulation by sugar were not investigated (Graham *et al.*, 1992). In maize root tips, MS but not ICL enzyme activities were detected in root extracts. The absence of ICL activities could be explained by the low level of enzyme activity, or because the enzymes were assayed only after 24 h glucose deprivation (Dieuide *et al.*, 1992). It is proposed that longer incubation time may be needed for the full induction of *Ms* and *Icl* expression in maize root tips or that improved methods may be necessary to detect the enzymes.

The expression of *Ms* and *Icl* genes in other plants or tissues has been shown to be coordinately regulated at the transcriptional level, but this is not always the case. In the root system, as shown in Figures 3.2 and 3.5, both *Ms* and



*Icl* genes are activated upon sugar deprivation showing the coordinate regulation, but the timing of MS mRNA accumulation is different from that of the *Icl* gene. One explanation could be that this difference reflects a different rate of transcription between the two genes. A similar situation has been observed in developing seeds of oilseed rape. Their mRNAs indicate similar qualitative but different quantitative patterns of accumulation during embryogeny and post-germinative growth (Comai *et al.*, 1989). Furthermore the enzyme activities or protein obtained do not reflect the amount of MS and ICL mRNA accumulated (Ettinger and Harada, 1990). In another case, the onset of MS and ICL synthesis in developing seeds occurred at different times (Turley and Trelease, 1990).

The induction of *Ms* and *Icl* genes in roots as well as in hairy roots of cucumber has confirmed and extended previous reports that their expression can be activated under appropriate conditions in many tissues. In addition to that, it indicates the possibility to further study the sequences through which sugar controls expression of these genes. Results presented in Figure 3.7 have revealed two important facts of *Ms* and *Icl* regulation. First it demonstrates that the control of *Ms* and *Icl* expression is apparently exerted primarily at the level of transcription. Second, the *Ms* and *Icl* promoter fused to GUS reporter gene is responsive in transgenic hairy roots under sugar deprivation, leading to the possibility of studying *cis*-acting sequences necessary for the expression of the genes in homologous transgenic hairy roots of cucumber, since a transgenic cucumber plant is yet to be generated.

Previous studies using heterologous transgenic plants or transient expression in electroporated cucumber protoplasts have identified *cis*-acting sequences necessary for sugar and germination responses in promoters of *Icl* and *Ms* genes (Reynolds and Smith, 1995b; Sarah *et al.*, 1996). It has been proposed that during carbohydrate deprivation, reduced metabolic flux through hexokinase

provides a signal which ultimately results in an activation of gene transcription (Graham *et al.*, 1994a; Jang and Sheen, 1994;). Further details of *Ms* and *Icl* promoter analysis using transgenic hairy roots will be discussed in the next chapter.

Fusion of the *Ms* or *Icl* promoter and the GUS reporter gene showed stable expression activity upon starvation in transgenic hairy roots. Surprisingly, GUS expression levels under the *Icl* promoter are much lower than those obtained for the *Ms* promoter GUS fusion. The possibility that the *Icl* promoter is intrinsically weak can be excluded, in view of the high transient and heterologous expression previously obtained with the same constructions (Reynolds and Smith, 1995b), as well as by the significant histochemical staining observed in the transgenic hairy roots (Figure 3.8 and 3.9 ).

As shown in Figure 3.6, the *Icl* promoter was fused to the 5'end of the GUS gene from pRAJ275 at the translation initiation codon ATG (Reynold and Smith, 1995b), whereas the fused junction between the 1076 bp *Ms* promoter and the 5'end of the GUS coding region does not contain the GUS initiation codon (Graham *et al.*, 1990), resulting in different GUS molecules with different activity or stability. In other circumstances, such as during seed development, a low level of non-coordinate expression of *Ms* and *Icl* can be detected, thus suggesting that variations of responsiveness of each gene may occur which is to be expected if each has its own promoter.

### **3.8.2. Carbohydrate regulation of gene expression *in planta***

In no cases has it been shown that ICL and MS are synthesised in whole plants under naturally occurring conditions in which carbohydrate deprivation is



induced. Results presented in Figures 3.10 and 3.11 show that shading and defoliation are both capable of reducing sugar content of roots and activating *Icl* and *Ms* gene expression, showing a correlation between the two rather than a demonstration of cause and effect. While the conditions employed to demonstrate this response appear relatively extreme, they may nevertheless be expected to occur frequently in nature.

The cucumber plants subjected to defoliation and shading grew perfectly well subsequently. There may be numerous other conditions which could also lead to carbohydrate deprivation of roots or other sinks, including pest and pathogen attack, mechanical damage, chemical exposure and photoinhibition due to UV-B. While individual conditions might not be sufficient to lead to extreme carbohydrate deprivation, they might do so in combination. Perhaps the most common situation in which carbohydrate deprivation occurs is during prolonged seedling growth beneath the soil (El Amrani *et al.*, 1994). The response to carbohydrate deprivation which includes synthesis of glyoxylate cycle enzymes could have evolved as a consequence of such factors. The evolution of this response implies that it has physiological significance. It is likely to occur in cultivated plants relatively infrequently since they do not normally experience such extreme conditions, but heavy grazing by farm animals, leaf cropping or pruning could induce synthesis. Furthermore, harvested plant products including leaf crops and fruits may experience carbohydrate deprivation leading to changes in product quality, partly through the activity of the glyoxylate cycle (Pistelli *et al.*, 1996).

# **CHAPTER FOUR**

## **RESULTS AND DISCUSSION**



## CHAPTER FOUR

### USE OF TRANSGENIC HAIRY ROOTS TO STUDY *CIS*-ACTING ELEMENTS IN THE *MS* AND *ICL* GENES

#### 4.1. Rationale

In the previous chapter, the expression of endogenous *Ms* and *Icl* genes in which sugar controlled transcription of genes in roots, was described. Both genes were activated in the absence of sugar but repressed when roots were fed with sugar. The fusions of *Ms* and *Icl* gene promoters to GUS were also capable of driving relatively high-level expression of the reporter gene in transgenic hairy roots. A previous study of the cucumber *Ms* gene demonstrated that its expression in transgenic plants is regulated at the transcriptional level under the control of DNA sequences in the *Ms* promoter (Graham *et al.*, 1990). Subsequently, analysis of *Ms* promoter deletions has separated distinct *cis*-acting elements in the promoter which are necessary for expression in germinating seeds, from those required for expression in response to sugar deprivation (Sarah *et al.*, 1996). However, both responses were assayed using transgenic *Nicotiana plumbaginifolia* plants since transgenic cucumber plants have not been obtained.

Sequences participating in both responses were also investigated in the *Icl* promoter, but a difference from the *Ms* promoter studies was that the sugar response was assayed using electroporated protoplasts of cucumber leaf. Expression of the *Icl* gene in germinating *Nicotiana* seeds and in response to sugar deprivation has been demonstrated; nevertheless the DNA sequences in its promoter required for each response were not distinguished. However, since *Icl* and *Ms* genes are coordinately regulated during germination and post-germination

as well as in carbohydrate deprivation, common sequences within the two promoters were expected.

Comparison of the two genes has led to the identification of conserved short DNA sequences contained within the *Icl* and *Ms* promoters which were referred to as IMH sequences (*Icl*-*Ms* Homology). It is suggested that they could have functions in the control of transcription of these genes. In this chapter, one aim was to establish if the *Icl* promoter contains distinct *cis*-acting sequences for the germination and sugar response as well as to seek evidence for possible function of one conserved DNA sequence in the sugar response. Since homologous transgenic hairy roots can be regenerated now, the second aim was to carry out the analysis of *Ms* promoter deletions in transgenic hairy roots in order to identify and confirm the *cis*-acting elements which participate in the sugar response which were previously investigated using heterologous *Nicotiana* plants (Sarah *et al.*, 1996) and cucumber protoplasts (Graham *et al.*, 1994b).



## 4.2. Localisation of *cis*-acting elements within the *Ms* promoter necessary for the sugar response in transgenic hairy roots

In a previous report, analysis of *Ms* promoter deletions identified DNA sequences believed to direct *Ms* expression in response to carbohydrate deprivation. In order to locate and confirm the region for this sugar response in the *Ms* promoter, the experiment was repeated using transgenic hairy roots.

In this experiment, the 1076 bp *Sau* 3A *Ms* gene promoter fused to GUS which was previously shown to direct GUS expression in transgenic *Nicotiana plumbaginifolia* (Graham *et al.*, 1990) was used. Subsequently this is referred to as MS1029 (Sarah *et al.*, 1996) since it contains 1029 bp MS promoter sequence upstream of the start of transcription (Graham *et al.*, 1989; 1990; 1994b). A series of deletions constructed from the 1076 bp gene fragment named MS248, MS233, MS216, MS199, MS182 and MS142 fused to GUS were subcloned into pBI101 as *Hind* III-*Eco*R1 fragments were used in order to localise the regulatory element(s) which control the expression (Sarah *et al.*, 1996).

These chimeric constructs were introduced into *Agrobacterium rhizogenes* and transferred into hairy roots by hypocotyl transformation of cucumber. Four independent transformant transgenic hairy roots were generated for each construct. Hairy roots were then subject to incubation in the dark for 6 days in the absence and presence of 25 mM sucrose, collected, the extracts prepared and GUS specific activity was determined. The GUS activity was measured in roots from each transformant and the mean activity for each construct is shown in Figure 4.1a. The results obtained in starved roots demonstrate that the highest levels of GUS activity are obtained from roots transformed with the 1076 bp *Ms* promoter.



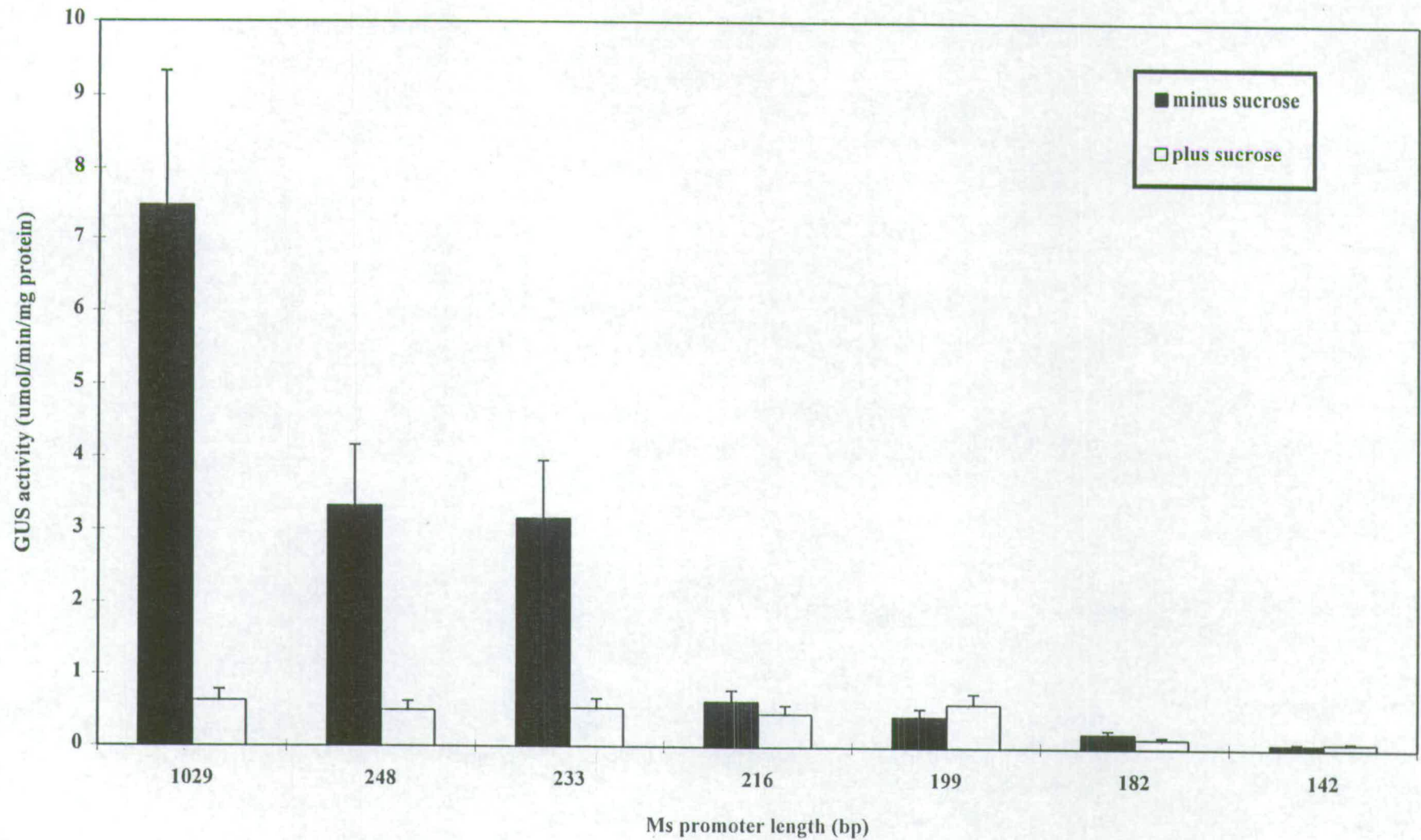


Figure 4.1 a) Sugar response of *Ms* promoter deletions in transgenic cucumber roots. Values presented are the average of the four independent transformants for each treatment. Standard errors (shown as error bars) are between 5 to 22% of the means.

Deletion of the *Ms* promoter to give rise to MS248 and MS233 results in a 50% decrease in GUS activity. The reduction in GUS activities directed by these promoters nevertheless does not reflect the deletion of the region necessary for the carbohydrate regulation of the cucumber *Ms* promoter. Deletion of a further 17 bp, resulting in the MS216 construct, causes a dramatic decrease in GUS activity. Furthermore, the MS199, MS182 and MS142 constructs do not direct significantly lower levels of GUS activity than is present in the MS216 construct. It is suggested that these constructs lack sequences necessary for the carbohydrate regulation of the cucumber *Ms* promoter. These results indicate that regulatory *cis*-elements involved in the quantitative levels of expression subject to sugar regulation reside between -233 and -216 bp of the *Ms* promoter.

In the presence of sucrose, GUS activities directed by MS1076, MS248 and MS233 shows no significant difference between one another. The pattern observed is qualitatively similar to that in *Nicotiana* transgenic seeds (Sarah *et al.*, 1996) in that the level of GUS activity is greater in the absence of sucrose for MS1029, MS248 and MS233. Repression by sucrose in each construct shown by the ratio of GUS expression levels in transgenic hairy roots cultured in the absence of sucrose to those cultured in the presence of sucrose is summarised in Figure 4.1b. The GUS activities in MS1076, MS248 and MS233 were repressed by sucrose between 7 and 13 fold. The repression is higher compared to similar assays carried out on the *Ms* promoter in mesophyll protoplasts where the GUS activity was repressed between 4 and 6 fold by sucrose (Graham *et al.*, 1994b). In contrast, the fold of repression is greatly reduced in MS216, MS 199, MS182 and MS142. Taking these data together, it confirms the finding that *cis*-acting sequences conferring sucrose responsiveness of the *Ms* promoter are located between 233 and 216 bp upstream of the transcription start. This shows that homologous transgenic hairy roots could be an excellent experimental system to study DNA sequences in which sugar controls the expression of genes.



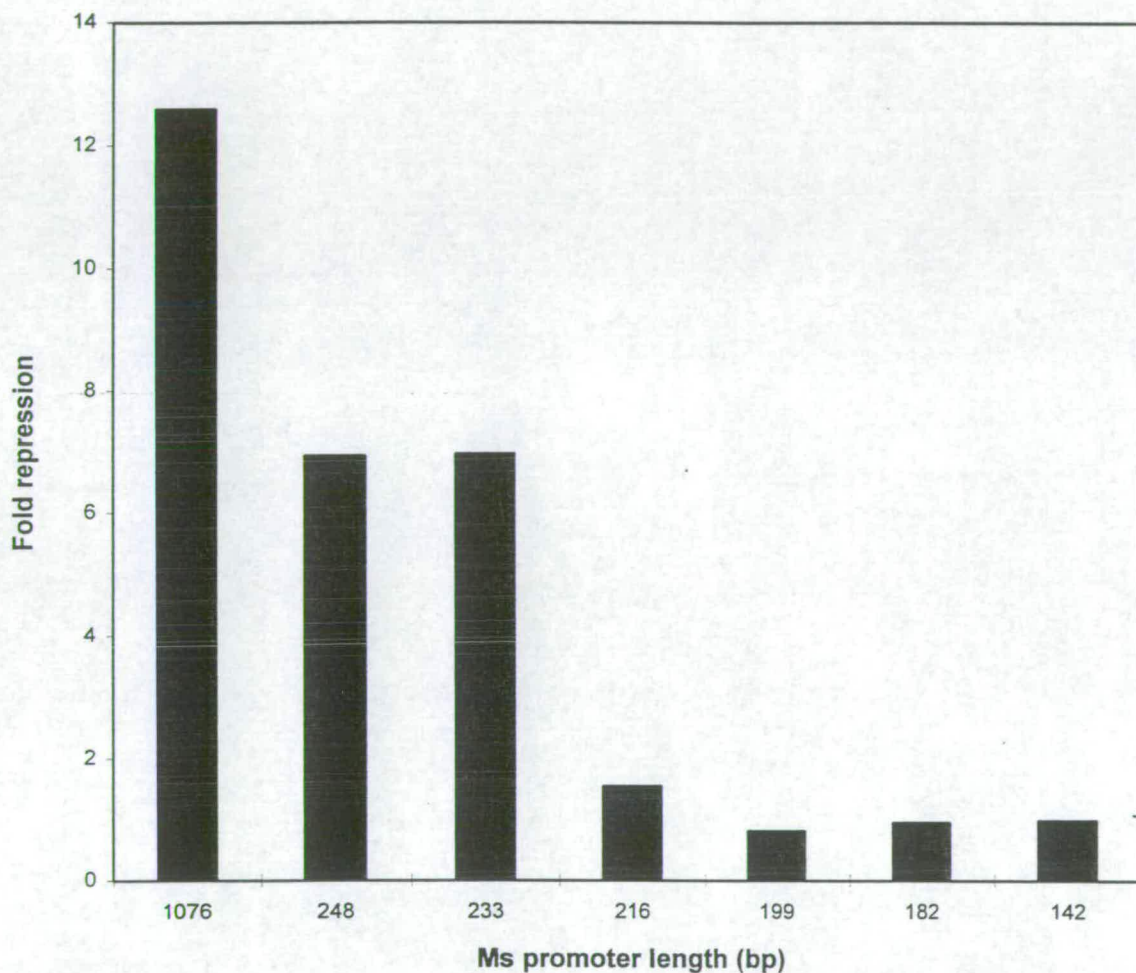


Figure 4.1b). Fold repression by sucrose. The GUS activity obtained in the absence of sucrose were divided by activity obtained in the presence of sucrose to give the fold of repression by sucrose. Values represent the average of the four independent transformants.

### **4.3. Analysis of 191 bp Alu1-Alu1 fragment in transgenic hairy roots**

The analysis of *Ms* deleted promoters in mesophyll protoplasts of cucumber has demonstrated that the region from position -125 to -248 bp is important in the sugar regulation of the *Ms* promoter. In order to further investigate this region, a fragment of 191 bp which spans from position -125 to -316 bp relative to the start of transcription was inserted in both the correct (one and two copies) and reverse (one copy) orientation in front of the core promoter (+8 to -46) of the CaMV 35S RNA gene fused to the GUS reporter gene (Graham *et al.*, 1994b). The results showed that both orientations give rise to a significant increase in GUS activities upon starvation, whereas two copies in the correct orientation results in even higher level of GUS activity (Graham *et al.*, 1994b). The same experiment was repeated in order to analyse this region using transgenic hairy roots.

### **4.4. Construction of a plant transformation vector containing the 191 bp fragment fused to the GUS gene**

These chimeric constructs were cloned into the binary vector pBIN19 (Bevan, 1984) for plant transformation, using the *Bam*HI site present in the polylinker of the plasmid ( Figure 4.2). The recombinant plasmids, identified by restriction endonuclease analysis, were transferred into *Agrobacterium rhizogenes* by a freeze thaw transformation. To transfer these chimeric genes into transgenic hairy roots, cucumber hypocotyl transformation was carried out as described in section 2.4.3. Transgenic hairy roots from 3 independent transformants for each construct were incubated on filter paper soaked either in water ( minus sucrose ) or in 25mM sucrose for 6 days in the dark.



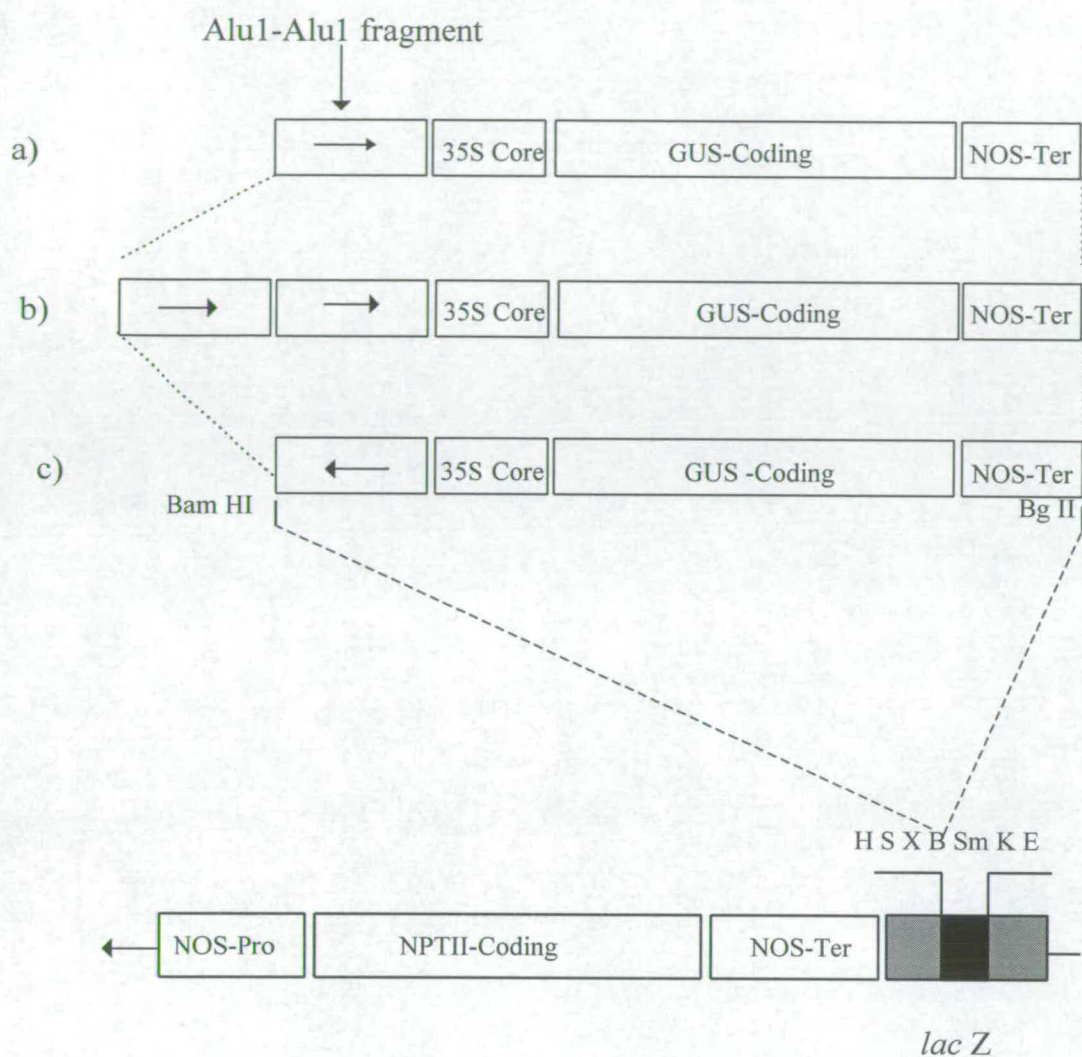


Figure 4.2 Construction of vector for plant transformation using 191 bp Alu1-Alu1 fragment. a) one copy, b) two copies and c) one copy with reverse orientation of Alu1-Alu1 fragment inserted in front of 35S core promoter fused to GUS (Graham et al., 1994b). These constructs were subcloned into binary vector pBIN19 (Bevan et al., 1984). NOS-Pro and NOS-Ter, nopaline synthase gene promoter and terminator; NPTII-Coding, neomycin phosphotransferase II coding region; *Lac Z*, fragment of galactosidase gene and polylinker site; H S X B S m K E, restriction sites in polylinker.

After incubation, extracts were prepared for the assay of GUS activity and protein content. The results are presented in Figure 4.3. One copy of the 191 bp fragment resulted in a significant amount of GUS activity when hairy roots were incubated in the absence of sugar but directed greatly reduced level of activity in the presence of sugar. In the reversed orientation, one copy of this sequence gave rise to slightly less GUS activity compared to correct orientation. Nevertheless, this difference is too small to be considered as the effect of reverse orientation. Two copies of this fragment in the correct orientation increased GUS activities almost two fold to that of a single copy, while maintaining the sucrose repression. Based on these data, three points can be made. First, the 191 bp AluI-AluI fragment is able to confer upon the core promoter a pattern of expression similar to that of the intact promoter in hairy roots but with no significant effect on the level of expression exerted by the reversed orientation. Nevertheless, the core promoter alone was not assayed. Secondly, more than a single copy of the fragment will induce a greater level of GUS activity. Finally, in all cases, the presence of sucrose greatly affects the action of these promoters, which results in a very low level of GUS activity. These results are consistent with those of Graham *et al.*(1994b) but show a greater fold repression by sucrose, again emphasising the value of the hairy root system.

#### **4.5. Identification of regulatory elements for the germination and sugar responses of the *Icl* gene**

Previously, the promoter of the *Icl* gene fused to the GUS reporter was employed to investigate its expression pattern in *Nicotiana* seedlings and electroporated protoplasts of cucumber. Two putative *Icl* promoters, 2900 bp and 572 bp, have been demonstrated to express GUS activity in *Nicotiana* following



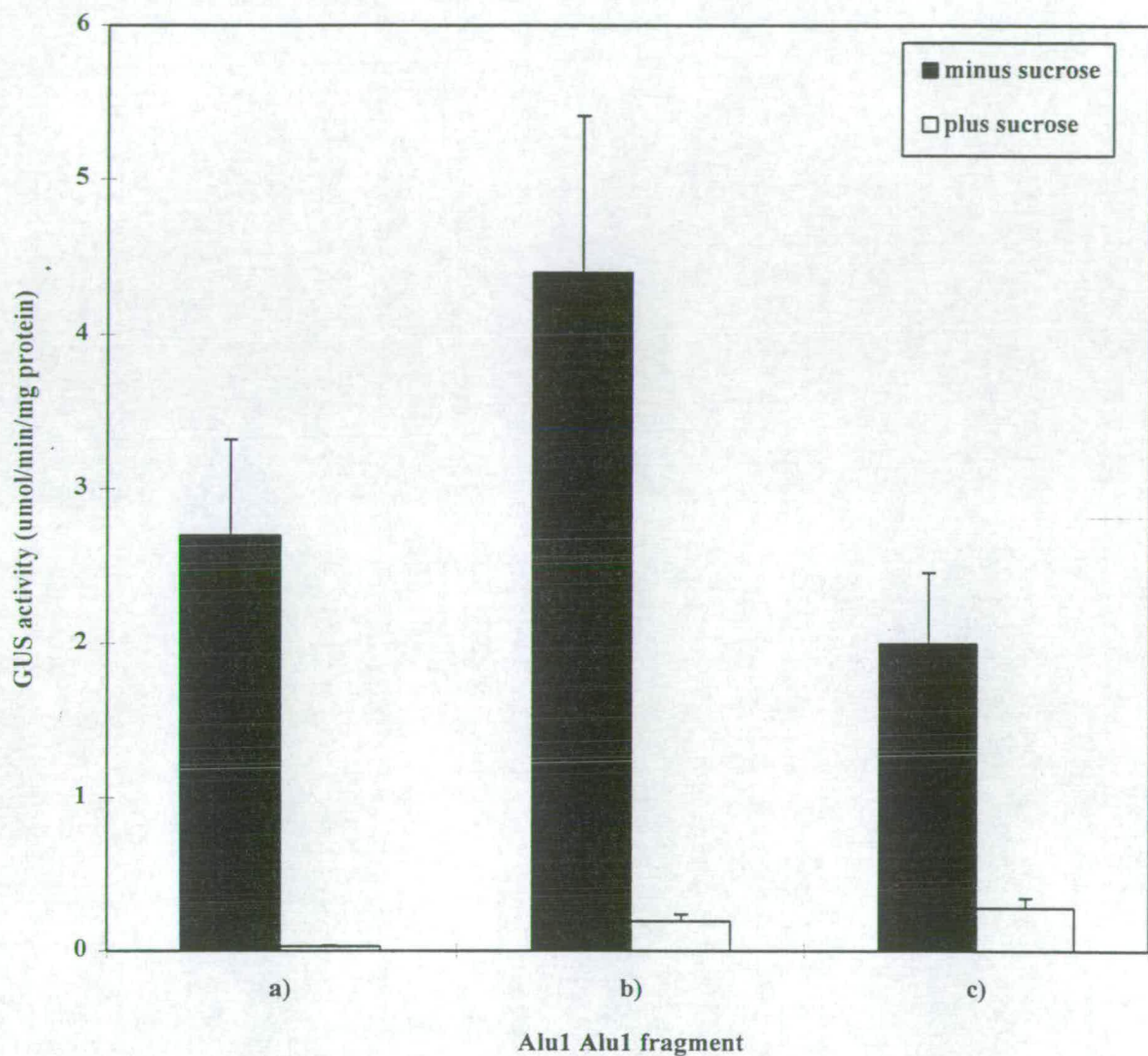


Figure 4.3 GUS activity of the 191 bp Alu1-Alu1 fragment of the *Ms* promoter fused to the core region of the CaMV 35S promoter in transgenic cucumber hairy roots. a) One copy, b) two copies in the correct orientation and c) one copy of the 191 bp fragment, in the reverse orientation. Standard errors (shown as error bars) ranged from 3 to 18% of the means, and values for one copy in the right (a) and reverse (c) orientation of Alu1 Alu1 fragment in the absence of sucrose are not significantly different to each other at the 95% confidence level.

seed germination, although the expression level in 572 plants was very much lower compared to that of 2900 plants. Both of these promoters preferentially direct the GUS expression in the cotyledons as shown by GUS histochemical staining, suggesting that they are organ-specific as previously shown for the *Ms* promoter (Reynolds and Smith, 1995b).

For the starvation response in seedlings, transferring seedlings from light to the dark causes activation of expression in 2900 plants in the absence of sucrose and repression upon addition of sucrose. However, 572 plants were not responsive. Hence, the *Icl* promoter directs both germination and sucrose response and implicates distinct *cis*-acting elements required for germination and sugar responses but information is lacking and needs further investigation. In order to resolve this region, several deletions were created, and a region between 1663 and 1142 bp (Figure 4.4a) upstream of the start of translation, was further defined which is required for the sugar response in cucumber leaf protoplasts (Reynolds and Smith, 1995b).

#### **4.6. Localisation of sugar-responsive region in *Icl* promoter**

Previous studies, using deleted *Icl* promoters in a transient assay system, had located a region between 1663 and 1142 bp in the *Icl* promoter responsible for the sugar response (Figure 4.4a). In order to further analyse this specific region, three more deletions were created within this region, each 1568, 1367 and 1193 bp upstream of the translational start, and fused to the GUS reporter (Figure 4.4b) (Drs. S.J. Reynolds and Luigi De Bellis, unpublished). These promoter fusions were subcloned into pBIN19 binary vector and were introduced into *Agrobacterium rhizogenes* strain A4 and used to inoculate cucumber hypocotyls in order to produce kanamycin-resistant hairy roots. Hairy roots containing these



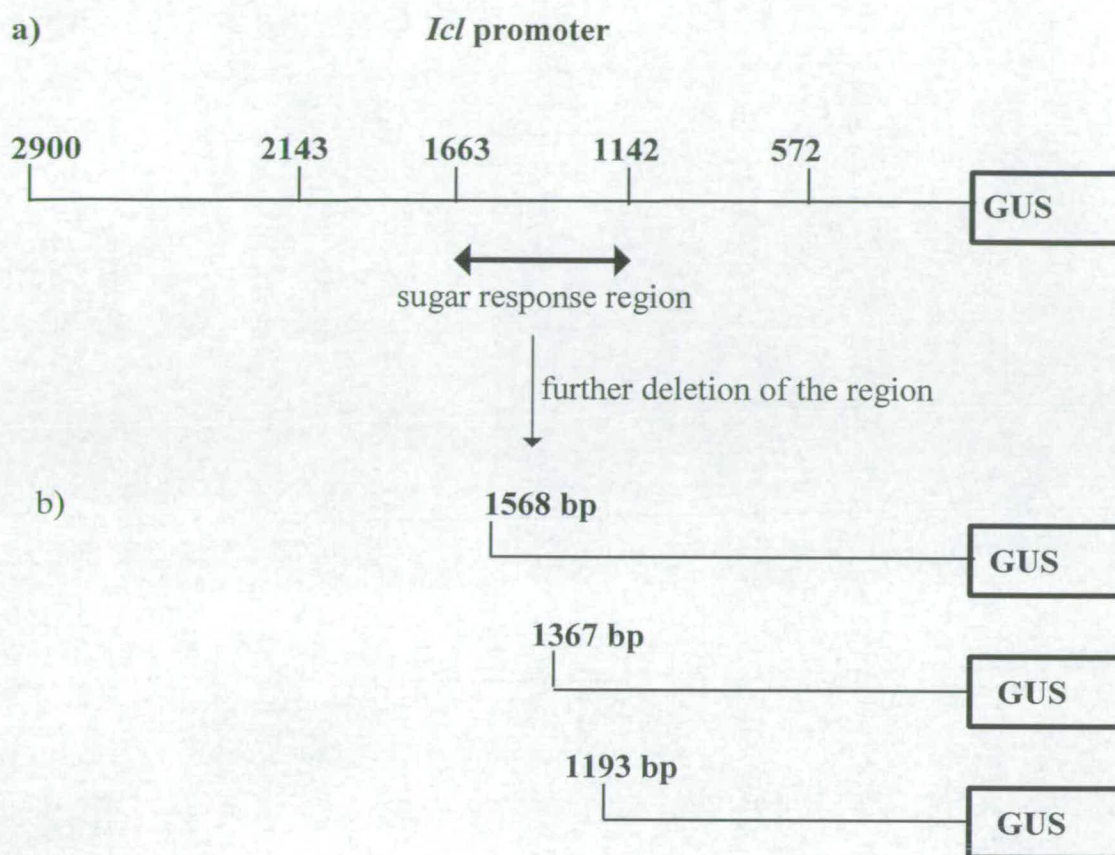


Figure 4.4 a). Schematic diagram of *Icl* promoter fused to GUS reporter gene showing the region between -1663 bp and -1142 bp required for the sugar response as reported previously (Reynolds and Smith, 1995b). b) Representation of further deletion series within the region fused to GUS (Luigi De Bellis, data unpublished). Numbering denotes the length (bp) of the *Icl* promoter sequence upstream of the start of translation.

constructs were grown and maintained as described in section 2.4.3. 1 cm sections of transgenic roots from 3 independent transformants for each construct, were incubated on filter paper soaked in water (without sucrose) or in 25mM sucrose for six days. After six days, roots were collected, and extracts prepared for the assay of GUS activity and protein content. The results are shown in Figure 4.5a.

The longest promoter (2900) directs the expression of GUS gene in the absence of sucrose, but this was repressed by addition of sugar to the incubation medium. The pattern and level of expression shown by the longest promoter in hairy roots agrees with those observed in *Nicotiana* seedlings and protoplasts (Reynolds and Smith, 1995b). Deleting the promoter to the position -1568 bp upstream of the translational start shows an activation in response to starvation, and higher level of repression by sucrose, which is about 10-fold. In dramatic contrast, further deletion to position -1367 or -1193 essentially abolishes the promoter activity in response to starvation. These results show that the 1568 bp promoter is sufficient to establish the quantitative level of expression and that the region between 1568 and 1367 bp upstream of translation contains *cis*-acting sequences necessary for the response to sugar deprivation (sugar response).

#### **4.7. Histochemical staining of hairy roots containing deleted *Icl* promoter**

Hairy roots from single representative transformants containing the *Icl* promoter deletions were incubated on filter paper soaked in water for six days, exactly the same treatment as for the GUS activity. After day six, the roots were stained with 5-bromo-4-chloro-3-indolyl- $\beta$ -glucuronide (X-gluc) and these are shown in Figure 4.5(b). GUS staining was observed in the hairy roots containing the 1568 bp promoter. The staining was found in all areas of the tissue and most



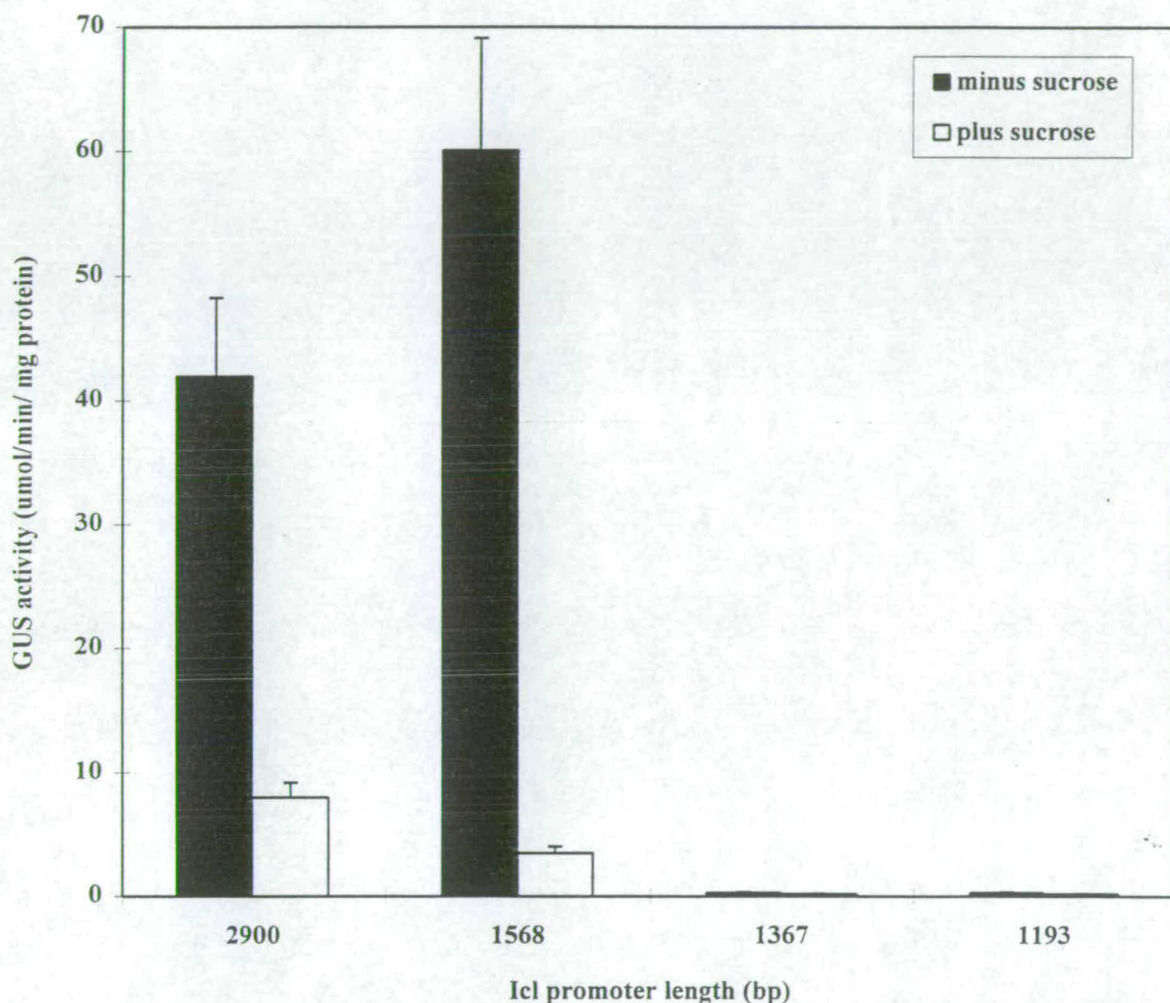


Figure 4.5. a) Sugar response of *Icl* promoter deletions in transgenic cucumber hairy roots. Value presented are the means of the same three independent transformants for each treatment. Standard errors (shown as error bars) are between 7 to 15% of the means, and values for 2900 and 1568 bp promoter in the absence of sucrose are not significantly different to each other at the 95% confidence level.

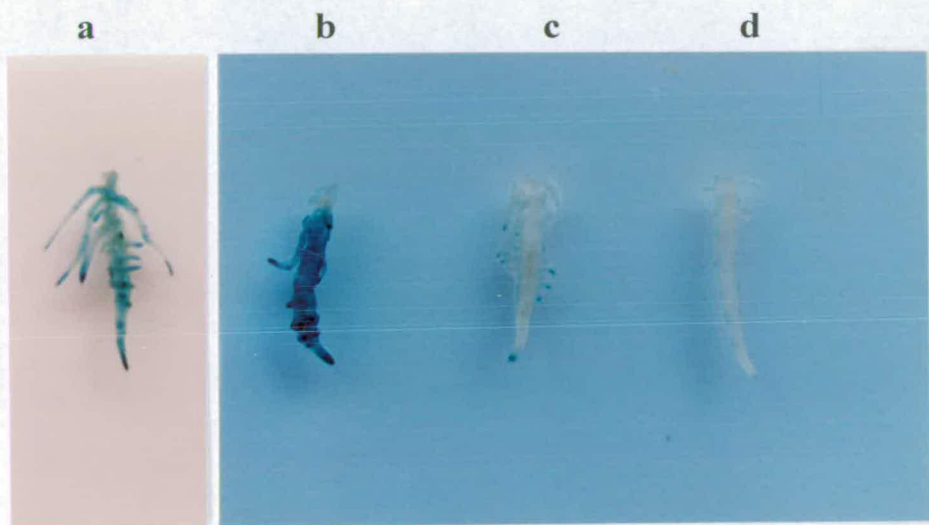


Figure 4.5. b) Effect of deletions on the histochemical staining of GUS activity directed by *lcl* promoter. These constructs are a). -2900 bp, b). -1568 bp, c). -1367 d). -1193bp relative to the translation start.

intense at the root tips. The pattern and level of GUS staining directed by 1568 bp promoter provides a direct comparison with those for the 2900 bp promoter shown in Figure 3.8. However, hairy roots transformed with the 1367 and 1193 bp promoters were hardly stained except blue spots observed at the root tips and a lesser extent at lateral primordia.

#### **4.8. Localisation of the germination-responsive region in the *Icl* promoter**

It has been shown that 2900 bp and 572 bp *Icl* promoter fusions direct GUS expression in germinating *Nicotiana* seeds although the 572 bp promoter gave rise to a very much lower activity compared to the 2900 bp promoter (Reynolds and Smith, 1995b). In order to locate the region within the *Icl* promoter required for the germination response, the same three constructs assayed in transgenic cucumber hairy roots were assayed in *Nicotiana plumbaginifolia* seeds and seedlings. Transgenic *Nicotiana plumbaginifolia* plants were used to assay germination response as they have been used successfully before (Graham *et al.*, 1990; Reynolds and Smith, 1995b).

The three constructs, 1568, 1367 and 1193 bp promoters fused to GUS, were transferred into *Nicotiana plumbaginifolia* using the leaf disc transformation system as described in section 2.5.1. (This work was initiated by Dr. L. Debellis and completed by the author). *Agrobacterium tumefaciens* carrying the recombinant plasmids containing *Icl* deletions were used to inoculate leaf pieces. Shootlets were formed and regenerated in the presence of kanamycin, and subsequently grew as entire plants which were grown to maturity, allowed to self-fertilise and generate seed. Six independent transformants were selected and seeds

collected. Seeds from each transformant were germinated and grown on M&S medium containing 400 µg/ml kanamycin in order to confirm that they were genuinely transformed. For GUS activity assay, the seeds were germinated on filter paper soaked in water without any antibiotic.

Transgenic *Nicotiana* seeds germinated in a non-synchronous manner. It was therefore necessary to analyse GUS activities at different stages of development rather than at certain time intervals after imbibition. Hence, seeds from all six independent transformants were germinated for at least five days during which seeds were collected at certain stages of their development. GUS activity was assayed in imbibed seeds (stage 0), in germinating seeds with maximal *Icl* gene expression (stage 4) and in green seedlings (stage 7) as depicted in Figure 4.6. (defined by Sarah *et al.*, 1996). The results (Figure 4.7a) show that the GUS activity in seed from transgenic plants containing the 2900 bp promoter increased from stage 0 to stage 4, but then declined again at stage 7, which is consistent with the previous report (Reynolds and Smith, 1995b).

The level of GUS activity showed a drastic reduction when the promoter was deleted to the position -1568 bp upstream of translational start. Therefore, it is proposed that sequences upstream of 1568 bp are necessary for high level expression of the *Icl* gene in the germination response. A small increase in expression appears in deletion -1367, and then declines significantly in deletion -1193. The increase is not understood but this could correspond to the presence of specific silencer between -1568 and -1367 bp or to variation among transgenic plants due to position effects. Although at a very low level of GUS activity, compared to the 2900 bp promoter, these *Icl* deletions maintained the pattern of expression throughout the three stages of plant development. Fluctuation in GUS activities among the deletions is not statistically significant, suggesting that all of them direct relatively similar quantitative patterns of expression in germinating



Figure 4.6 *Nicotiana plumbaginifolia* seedlings at the different developmental stages 0 (imbibed seed), 4 (germinating seeds with maximal *Icl* gene expression) and 7 (green seedling) following the onset of germination. Seeds were imbibed overnight at 4°C in 1 mM gibberellic acid and germinated on filter paper soaked in water without any antibiotic for five days during which seeds were collected at stages as above.



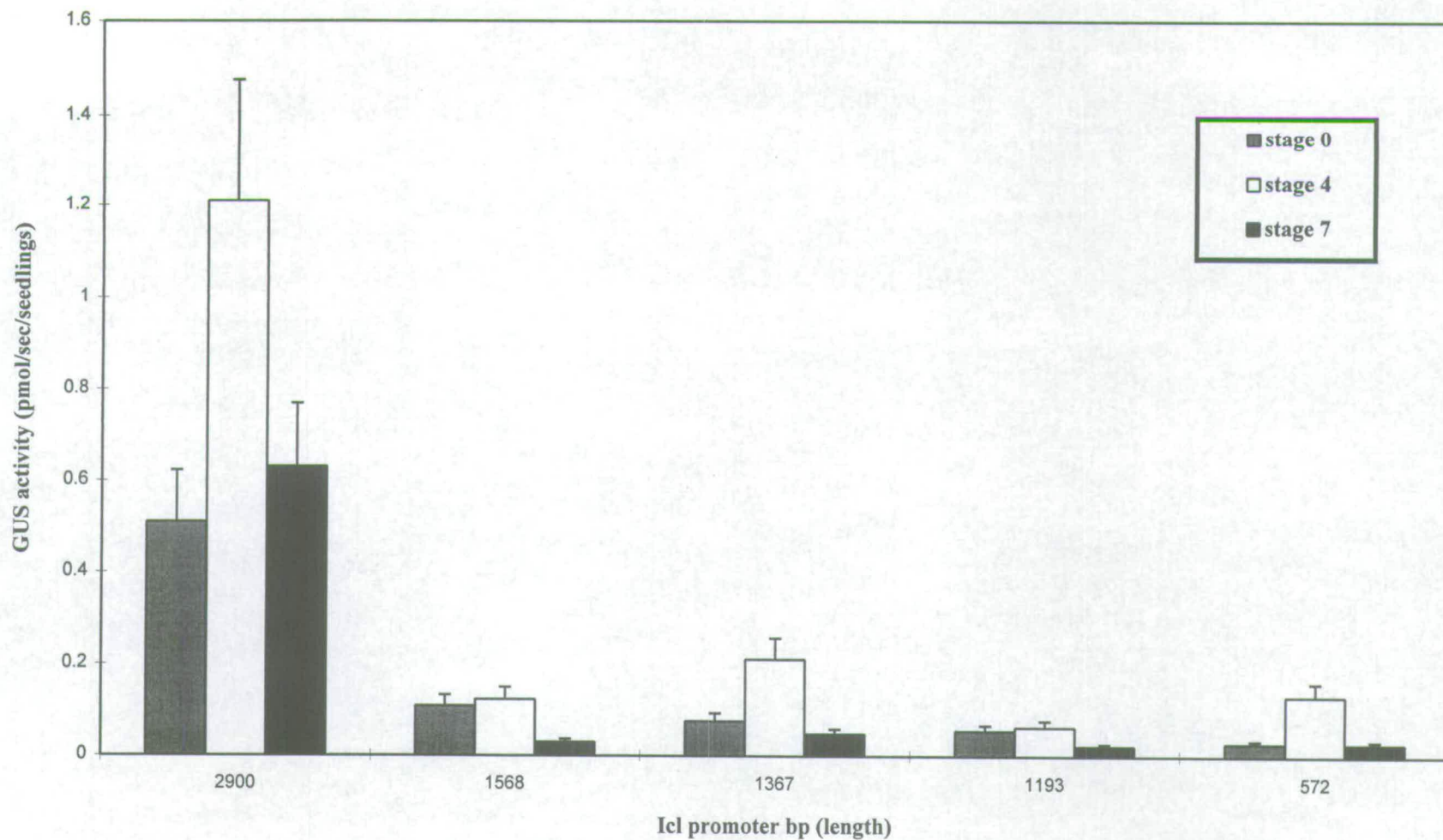


Figure 4.7a). Germination response of the *Icl* promoter deletions in transgenic *Nicotiana* seeds. Values represent the means of the six independent transformants. Standard errors (shown as error bars) are between 12 to 22% of the means.



seeds. In addition to that, the shortest promoter (572 bp) was previously reported to have enough information to direct *Icl* gene expression but at a very low level (Reynolds and Smith, 1995b).

#### **4.9. Effect of 5' deleted *Icl* promoter on organ specific expression**

In order to further monitor gene expression and visualise cells or tissues expressing the fusion constructs, histochemical assays for GUS activity were carried out. In transgenic germinating seedlings of *Nicotiana*, 572 bp upstream of the translation start preferentially induced GUS expression in the cotyledons which are the main storage reserve of the seed and a much lesser extent at the root tip (Reynolds and Smith, 1995b). In order to investigate the effects of 5' promoter deletions on the organ specific pattern of *Icl* promoter activity, transgenic *Nicotiana* seedlings were histochemically stained for GUS activity. Transformed *Nicotiana* seeds of each fusion together with 2900 and 572 bp promoters were germinated for five days. As the seedlings always grow in a non-synchronous manner, seedlings reaching stage 4, which shows high level of GUS activity, were collected for GUS staining. The testa and endosperm were carefully removed to avoid any damage to the seedlings which were subsequently incubated in 1 mg ml<sup>-1</sup> X-gluc for 1 h at 37°C (section 2.6.3) .

As shown in Figure 4.7b, transformed *Nicotiana* seeds containing the longest 2900 bp promoter show intense blue colouration but this is lacking in seedlings containing deleted promoters. This result corresponds to the level of GUS activities shown in Figure 4.7a. Seedlings containing *Icl* deletions were left overnight for better staining in order to investigate the effect of deleted *Icl* promoter on organ specific expression. Seeds from two independent transformants

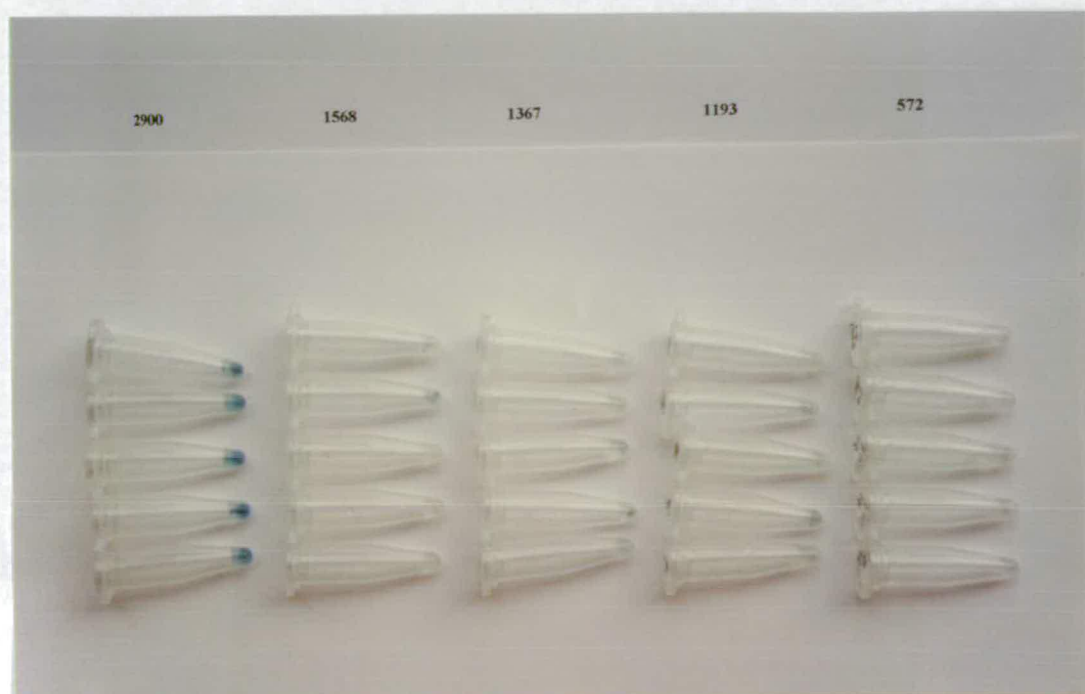


Figure 4.7 b) Corresponding histochemical staining of GUS activity in five independent transformants of each *Icl* construct. Each tube contains 10 seedlings (stage 4) and were stained for 1 h.



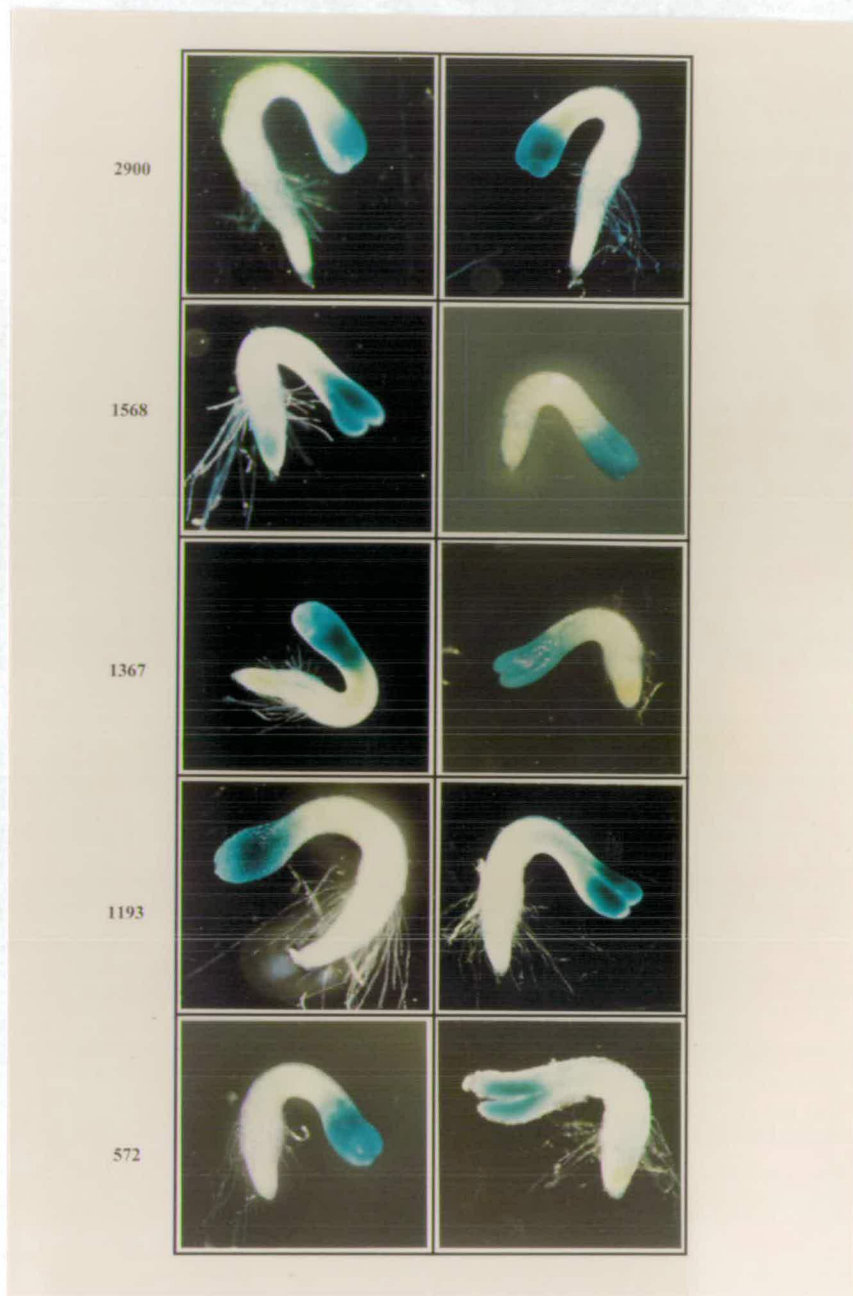


Figure 4.8 Histochemical localisation of GUS activity within transformed *Nicotiana* seeds. Seeds from plants transformed with the *Icl* constructs were germinated as indicated and stained for glucuronidase activity. 2900 seedlings were stained for 1 h, whereas 1568,1367, 1193 and 572 seedlings were stained overnight.

of each construct were chosen and the result is shown in Figure 4.8. 2900 and 572 bp promoters cause the correct spatial patterns of expression in cotyledons, just as previously reported (Reynolds and Smith, 1995b). Although the staining intensity in different seedlings was found to be quite variable among transformants for each construct (data not shown), no significant difference was found in the organ specific pattern of GUS expression between seedlings containing the longest promoter (2900) and its deletions. Thus, seedlings containing the shorter promoters still induced GUS expression in the cotyledons, as seen for seedlings transformed with the *Icl* gene containing the longest promoter. In addition, blue staining was also observed at the root hairs of the seedlings.

## **4.10. Discussion**

### **4.10.1. Homologous hairy roots of cucumber as an alternative system to study cucumber *Ms* gene expression**

In this chapter, hairy roots produced by hypocotyl transformation using *Agrobacterium rhizogenes*, were used to characterise the regulatory elements responsible for sugar response, previously investigated using transgenic *Nicotiana* seedlings. Deletion analysis of the *Ms* promoter in *Nicotiana* (Sarah *et al.*, 1996) has shown a region between -233 and -216 bp relative to the start of transcription that appears to be required for the sugar response. Nevertheless, the results obtained do not show conclusively whether DNA sequence located within this region direct the *Ms* expression upon sugar starvation. It is seen that the GUS activity decreased about 4-fold when the *Ms* promoter was deleted to position -248 bp relative to the transcription start. About the same fold decrease was observed when further deletion was made to the position -233. Even though the GUS

activity is very low in -216, the difference with that of -233 is very small (Sarah *et al.*, 1996)

The experiment was repeated, but this time using homologous transgenic hairy roots. It should be stressed here that the step taken was not to dispute the previous result but rather to look at the effectiveness of hairy roots as a better alternative system to study *Ms* gene expression and subsequently strengthen and support such findings. The results shown in Figure 4.1(a) confirm the findings of Sarah *et al.* (1996) which indicated the region between -216 and -233 bp relative to the start of *Ms* promoter appears to be required for the sugar response. The GUS activity in the -248 bp promoter reduced by 50% compared to the longest promoter and was maintained in the -233 bp promoter before the level dropped dramatically in the -216 bp fragment, indicating a loss of promoter activity to direct expression upon starvation. Looking at the fold of repression, (Figure 4.1b) the value also decreased drastically in the 216 bp deletion and was maintained subsequently in further deletions.

The effectiveness of homologous hairy roots was also tested with the 191 bp Alu1-Alu1 fragment analysis previously shown to confer *Ms* expression similar to that of the intact MS promoter in protoplasts (Graham *et al.*, 1994b). The pattern of expression of one and two copies of the 191 bp fragment and reversed orientation agree very well to those in the cucumber protoplast system. The only difference noted between the two systems is the degree of repression by sucrose. While the protoplast system demonstrated only 40-50% repression, in the presence of sucrose in each case, the homologous hairy roots showed very much higher levels of repression. Taking these data together, it is suggested that hairy roots of cucumber are a superior system to heterologous *Nicotiana* plants or electroporated protoplasts of cucumber to study DNA sequences through which sugar controls the transcription of these genes.

#### **4.10.2. Distinct DNA sequences are required for the *Icl* promoter in germination and sugar responses**

Recent data have shown that distinct *cis*-acting elements direct the germination and sugar responses of the cucumber *Ms* gene (Sarah *et al.*, 1996). However, in the *Icl* gene which is coordinately regulated with *Ms*, the information is lacking even though both germination and sugar response were demonstrated previously in transgenic *Nicotiana* plants and electroporated cucumber leaf protoplasts, respectively (Reynolds and Smith, 1995b). Analysis using transient expression, revealed the region between -1663 and -1142 bp relative to the translation start is necessary for the sugar response (Figure 4.4a). In order to further define this region in the *Icl* promoter, deletions from the 5' end of the *Icl* promoter were created (S. J. Reynolds and De Bellis, pers. comm.) within this region (Figure 4.4b) and introduced into the *Nicotiana* plants and cucumber hairy roots. The results presented here have clearly demonstrated that the sugar regulation of *Icl* gene expression is separated from developmental regulation during seed germination.

Analysis of expression in transgenic hairy roots of cucumber shows that both 2900 bp and 1568 bp promoter are capable of directing a strong sugar response while further deletion to -1367 and -1193 bp dramatically reduced this response. For the germination response in *Icl* genes, deletion to position -1568 bp relative to the translation start has caused a drastic loss in promoter function. The crucial observation of these results is that the 1568 bp promoter is capable of directing an apparently normal sugar response, but not the germination response. Therefore, it is proposed that sequences upstream of -1568 bp are required for the germination response, while sequences between -1568 and -1367 bp are required for the sugar response. These results established that *Icl* and *Ms* genes are similar in that they both contain distinct *cis*-acting sequences in their promoters for sugar



and germination responses. Expression of some other plant genes (Grierson *et al.*, 1994; Rocha-sosa *et al.*, 1989) is also mediated by separate *cis*-acting sequences for metabolic and developmental regulation.

However, previous analysis of the germination response in transgenic *Nicotiana* plants showed that the 572 bp promoter is able to direct temporal and spatial patterns of expression in cotyledons, but at a very reduced level (Reynolds and Smith, 1995b). Similar results have been obtained from the GUS histochemical staining of seeds containing 1568, 1367 and 1193 bp promoters which also show the correct temporal and spatial pattern of expression in cotyledons (Figure 4.8). These data implicate different interacting *cis*-sequences within the region necessary for the germination response. The result of the *Icl* promoter deletion analysis, can be summarised in the form of organisation of sequences in the *Icl* promoter as shown in Figure 4.9(a). This diagram clearly shows the various *cis*-acting sequence elements within the *Icl* promoter which respond to two different environmental signals, sugar and germination response.

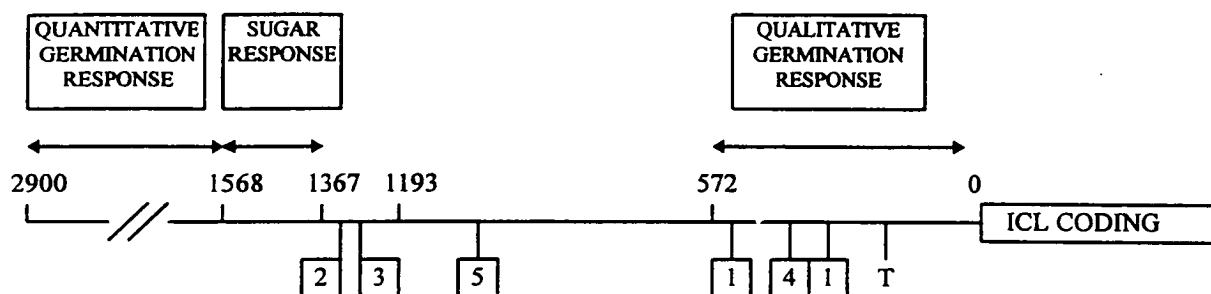
Besides the sugar response region, two separate *cis*-acting sequences required for directing the germination response are suggested, which is an interesting observation. Sequences downstream of the sugar response region direct a low level germination response (so called qualitative response) but sequences further upstream are required for a quantitative germination response. There is no evidence for a similar separation of such quantitative and qualitative germination response sequences reported in the *Ms* promoter (Sarah *et al.*, 1996). These results suggest that the temporal, spatial and sugar dependent *Icl* gene expression resulting from deletion analysis of the *Icl* promoter can be understood by an interaction of different *cis*-acting elements and their corresponding factors.

#### 4.10.3. Gain of function assay: An alternative to analyse *Ms* and *Icl* gene expression

Analysis of the regulatory properties of 5' promoter deletions of *Ms* and *Icl* genes using transgenic *Nicotiana* plants and electroporated protoplasts of cucumber (Graham *et al.*, 1990; Sarah *et al.*, 1996; Reynolds and Smith, 1995b; Graham *et al.*, 1994b) has enabled certain regions within the promoter to be identified and their function postulated. Furthermore, in this project, the production of cucumber hairy roots using *Agrobacterium rhizogenes* has allowed further analysis of the genes to take place in a homologous system. Using this system, it has been shown that metabolic and developmental responses appear to be regulated by distinct *cis*-acting elements, and independent regions within the *Icl* promoter (Figure 4.9a) could be controlled by two signal transduction pathways. These results have extended, and proved to be consistent with the previous findings.

Previously, comparison between *Ms* and *Icl* genes revealed a number of sequences showing similarity, which have been termed as IMH sequences (Reynolds and Smith, 1995b) and their location within *Icl* promoter is shown in Figure 4.9(a). One of these conserved DNA sequences (IMH2) (Figure 4.9b) was postulated to have a role in the sugar response. Results presented here show that the region between -1568 and -1367, necessary for sugar response, does not contain IMH2. But, this conserved sequence is located a few bases downstream of the -1367 bp promoter (Figure 4.9a). Therefore, it is suggested that IMH2 requires sequences further upstream to be able to respond to the metabolic signal. Further scrutiny of *Ms* and *Icl* promoters suggests that the homology between IMH2 sequences is probably more extensive than the previously reported 11 bp and could

a)



b) IMH2:

*Icl* - 1158 (1348) AAcCCCAaCCT -1148 (1338)

*Ms* - 227 (266) AAaCCCAcCCT -217 (256)

Figure 4.9 a). Anatomy of the *Icl* promoter. The promoter region is represented as a horizontal line, with distances upstream from the translation initiation codon (0) shown in bp above the line (2900,1568, 1367, 572). These distance correspond to the promoter deletions which have been studied. Numbers in boxes below the line show the position of IMH sequences (Sarah *et al.*,1996). T is the transcription start. Descriptors in large boxes above arrows, show functional regions of the *Icl* promoter. b) Conserved DNA sequences of IMH2 in *Ms* and *Icl* gene. Numbers indicates distances upstream of transcription start (not in parentheses) and translation start (in parentheses) Bases are shown in upper case when the same in both genes.

extend further upstream.

However, to further define and understand the sequences necessary for the sugar response, it requires gain of function assay rather than deletion studies as shown here. So, the next experiments were designed to establish if any of the IMH sequences can function to confer sugar responsiveness on the 35S core promoter. Having known that IMH1, IMH2 and IMH5 may confer metabolic and germination responses (Sarah *et al.*, 1996), four copies of these IMH's were created on their own, as well as a PCR product including IMH2, IMH1 and IMH5 together (Dr. Aideen Gill, data unpublished). These regions were then inserted in front of the core promoter linked to the GUS reporter gene and subsequently subcloned into the pBIN19 binary vector (figure 4.10). Numerous attempts to introduce these constructs into cucumber hairy roots were made without success. One reason for this was thought to be a supply of M&S medium (purchased in January 96) which was later reported to be defective (C.J. Leaver, pers. comm., September 96). Despite replacing this M&S with a supply from a different company, some improvement in root growth was noted but no new transgenic roots could be obtained in the time available. Once this problem is overcome, a detailed analysis of putative sugar-response sequences is possible through gain of function assays.

# **PSKBT2**

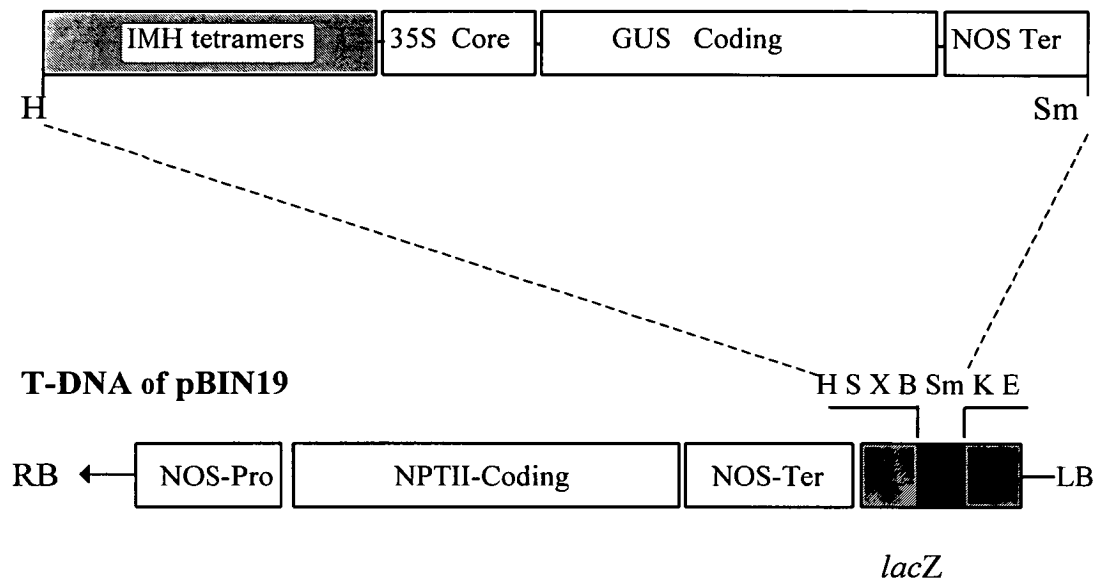


Figure 4.10. PSKBT2 plasmid with IMH tetramers inserted upstream of 35S core promoter fused to GUS (Aideen Gill, data unpublished). The IMH constructs were then isolated and subcloned into binary vector pBIN19 (Bevan, 1984). IMH representing IMH1, IMH2, IMH5 and IMH215. RB and LB, T-DNA right border and left border; NOS-Pro and NOS-Ter, nopaline synthase gene promoter and terminator; NPTII-Coding, neomycin phosphotransferase II coding region; *lacZ*, fragment of  $\beta$ -galactosidase gene and polylinker site; HSXBSmKE, restriction sites in polylinker.

## **CHAPTER FIVE**

### **RESULTS AND DISCUSSION**



# CHAPTER FIVE

## SUGAR EFFECT ON THE *MS* AND *ICL* GENE

### EXPRESSION AT THE LEVEL OF mRNA STABILITY

#### 5.1. Rationale

The importance of post-transcriptional regulation of gene expression has become increasingly apparent recently. Among these processes, one that has begun to receive considerable attention is the control of mRNA stability. Studies carried out in diverse organisms (Peltz *et al.*, 1991; Gallie, 1993; Green, 1993; Morris *et al.*, 1993) have shown an important role of mRNA degradation, which can have a profound impact on gene expression. The rate of mRNA degradation can vary widely among different mRNAs. In *E. coli*, mRNA half-lives vary over at least a 50-fold range from seconds to 25 min (Nilsson *et al.*, 1984) and are affected in a variety of host mutants (Ono and Kuwano 1979; Donwan and Kushner, 1986). Eukaryotic cells display a wide range of stabilities with half-lives ranging from min to days. For example, protooncogene mRNAs are typically unstable with half-lives of about 10-30 min (Laird-Offringa, 1992). Longer half-lives of greater than 24 h can be observed in  $\beta$ -globin mRNA in erythroid cells (Ross and Pizarro, 1983).

The fact that the decay rate of many mRNAs is subject to regulation provides a powerful means for controlling gene expression during physiological transition. A wide variety of endogenous and environmental stimuli have been found to affect the decay. Excess of iron tends to destabilise transferrin receptor mRNA in mammalian cells (Casey *et al.*, 1988) and heat shock destabilizes  $\alpha$ -amylase mRNA in gibberellin-treated barley aleurone. In addition to that, metabolic status has also been shown to affect mRNA stability. An experiment

which used actinomycin-D as a transcriptional inhibitor demonstrated that under sucrose starvation,  $\alpha$ -amylase mRNA in suspension cultured rice cells is more stable but destabilisation of the mRNA takes place upon addition of sucrose into the medium (Sheu *et al.*, 1994)

Studies so far have focused on the transcriptional regulation of *Ms* and *Icl* gene expression. There are numerous potential components of signal transduction pathways which may mediate such control and other potential mechanisms which may regulate gene expression. Studies on the rice  $\alpha$ -amylase gene which is also repressed by sugars and activated by starvation have provided clues about possible signal transduction components involved in the control of gene expression (Yu *et al.*, 1992). Other studies also on the  $\alpha$ -amylase gene have indicated that okadaic acid and calyculin A, potent and specific inhibitors of protein serine/threonine phosphatases 1 and 2A, strongly induce gene expression. Therefore, these results suggest a role for protein phosphorylation in the control of  $\alpha$ -amylase gene expression (Lue *et al.*, 1994). One aim was therefore to determine if *Ms* and *Icl* gene expression responded in the same way as  $\alpha$ -amylase to such inhibitors thus to establish if these genes all respond through a common signalling pathway.

Furthermore, other experiments with the  $\alpha$ -amylase gene indicated that mRNA stability was affected by sugars. Addition of actinomycin-D to stop transcription, followed by incubation in the absence and presence of sugar, showed that  $\alpha$ -amylase mRNA had a half-life of 12 h in the absence of sugar but only 1 h in the presence of sugar. In addition, cycloheximide, a protein synthesis inhibitor, caused mRNA accumulation in the presence of sugar (Sheu *et al.*, 1994). One interpretation of this result is that mRNA turnover requires newly synthesised protein. It was therefore decided to determine if similar phenomena regulate expression of *Ms* and *Icl* genes.

## 5.2. Effect of okadaic acid on derepression of *Icl* gene expression

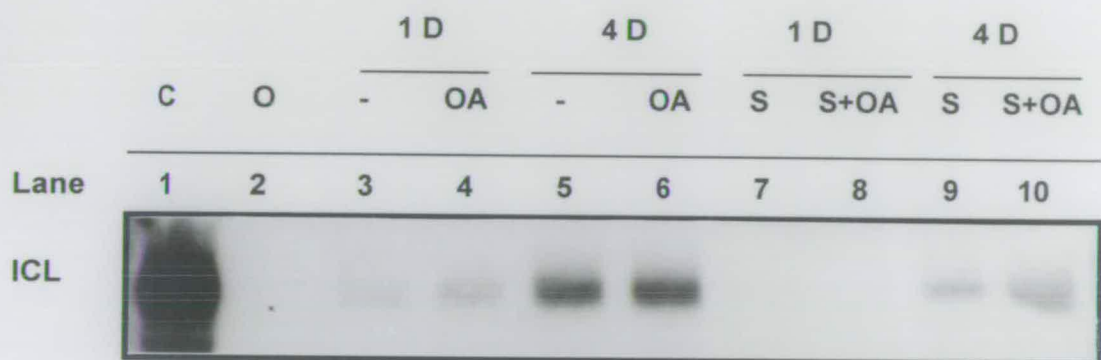
Previous results had demonstrated that okadaic acid induced  $\alpha$ -amylase mRNA in the presence of sugar, suggesting a role of protein phosphorylation in regulating gene expression (Lue *et al.*, 1994). A similar experiment was therefore carried out to investigate if okadaic acid has an effect on *Icl* derepression. Cucumber seeds were imbibed at 4°C in sterile distilled water and subsequently germinated for 5 days. After this period, roots were excised (day 0) and incubated for 1 and 4 days in the presence and absence of sucrose and in the presence and absence of 400 nM okadaic acid. Roots at day 0 were collected and kept at -70°C. After such intervals, roots were collected, RNA was isolated and subjected to northern blot using the *Icl* cDNA probe.

The result is shown in Figure 5.1. After 1 and 4 days incubation in water, the level of ICL mRNA increased to a significant amount (lane 3 and 5), whereas in the presence of sucrose, ICL mRNA accumulation was inhibited (lane 7 and 9). Addition of okadaic acid appears to have no significant effect on derepression of *Icl* expression. The level of ICL mRNA in okadaic acid treated roots was approximately the same as in roots incubated in water. This is in marked contrast to the rice  $\alpha$ -amylase gene in rice suspension-cultured cells which showed that okadaic acid strongly and rapidly induced  $\alpha$ -amylase mRNA accumulation (Lue *et al.*, 1994).

## 5.3. Effect of cycloheximide on derepression of *Ms* and *Icl* gene expression

To analyse the effect of the translation process on derepression of *Ms* and *Icl* gene expression, roots were treated with 200  $\mu$ M cycloheximide, a protein

Figure 5.1 Effect of okadaic acid on ICL mRNA accumulation. Roots were excised (O) and incubated for 1 and 4 days in the presence (S) and absence (-) of sucrose and in the presence (OA) and absence (-) of okadaic acid. RNA was isolated and subjected to northern hybridisation. Lane 1: RNA from cotyledons (C) of germinating seeds. 10 µg of total RNA was loaded per track.



synthesis inhibitor which blocks polypeptide chain elongation. After 5 days of seed germination, cucumber roots were excised and incubated for 6, 12, 24, and 48 h in water or sucrose containing 200  $\mu$ M cycloheximide. Total RNA was isolated and then subjected to northern blot hybridisation using the *Ms* cDNA probe. A replicate gel was hybridised with the *Icl* probe.

Results are shown in Figure 5.2. In the absence of sucrose (lanes 3-6) the level of mRNA increases even in the presence of cycloheximide. Nevertheless, in the presence of sucrose, addition of cycloheximide did not cause accumulation of mRNA (lanes 7-10) showing no effect on derepression of both genes, in contrast to previous results with rice  $\alpha$ -amylase (Sheu *et al.*, 1994). A similar result was obtained using the *Icl* cDNA probe.

## **5.4. Effect of mRNA stability on *Ms* and *Icl* gene expression**

### **5.4.1. MS and ICL mRNAs decrease in the presence of sucrose**

Previous experiments have examined the *Ms* and *Icl* gene expression in roots over a period of days. In order to further investigate the effect of sucrose on the steady state level of MS and ICL mRNAs, detached roots were starved for 4 days to accumulate high levels of MS and ICL mRNAs, then incubated in the absence and presence of 25 mM sucrose for 4 h. RNA isolated from the roots was then subjected to northern hybridisation using the *Ms* cDNA probe.

The result (Figure 5.3) shows that after 4 h sucrose treatment (lane 4), the mRNA level has declined dramatically whereas in the absence of sucrose, the mRNA level remains high (lane 3). This establishes that detached cucumber roots respond similarly to callus cultures which showed that addition of glucose to



Figure 5.2 Effect of cycloheximide on MS and ICL mRNA accumulation. Roots were excised (O) and incubated with 200  $\mu$ M cycloheximide in the presence and absence of sucrose. RNA was isolated and subjected to northern hybridisation. Lane 1: RNA from cotyledons (C) of germinating seeds. 10  $\mu$ g of total RNA was loaded per track.

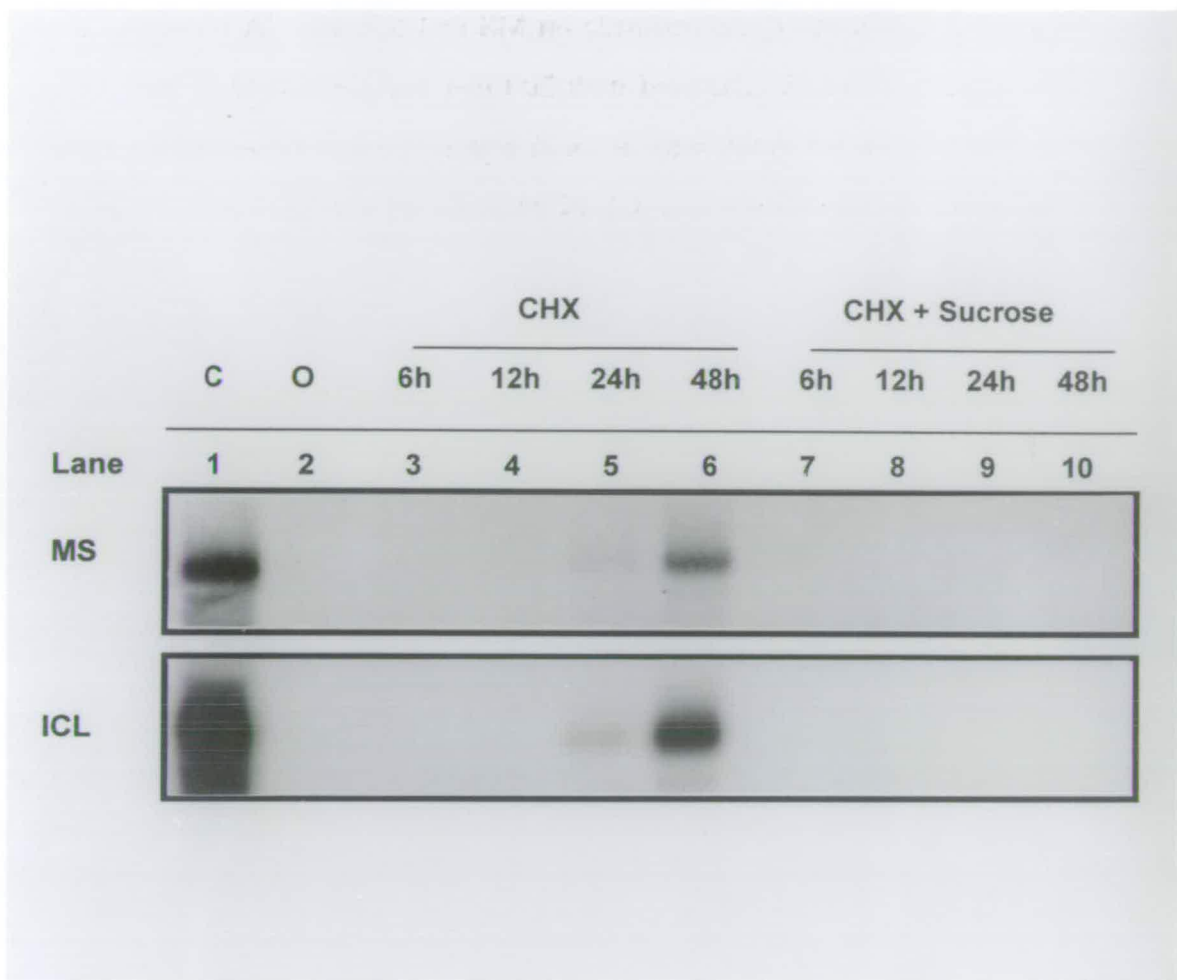
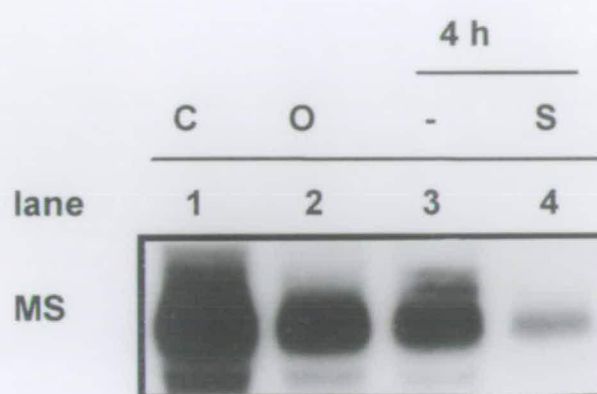


Figure 5.3 Effect of sucrose on MS mRNA accumulation. Roots were excised and starved for 4 days (O), followed by incubation for 4 h in the presence (S) and absence (-) of sucrose. RNA was isolated and subject to northern hybridisation. Lane 1: RNA from cotyledons (C) of germinating seeds. 10  $\mu$ g of total RNA was loaded per track.





previously starved cultured cells resulted in the loss of MS and ICL mRNAs within 4 h (Graham *et al.*, 1994a).

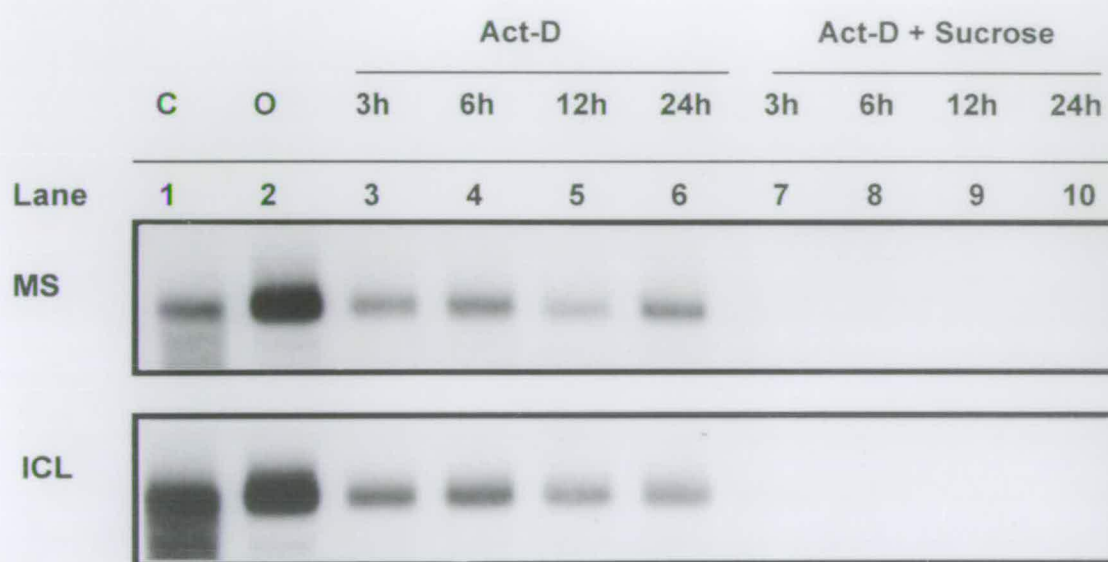
#### 5.4.2. Effect of sucrose on MS and ICL mRNAs stability

The previous experiment has shown that a period of a few hours is appropriate for further experiments to investigate the stability of MS and ICL mRNAs. The rationale for the approach was based on similar experiments carried out by Sheu *et al.* (1994). In this experiment it was shown that accumulation of  $\alpha$ -amylase was apparent after 48 h of sugar starvation, but then decreased significantly within 2 h after sucrose was added to the cells. The next experiment was set up to determine if there is any evidence for a difference in stability of MS and ICL mRNAs in the presence and absence of sucrose. The idea was to induce MS and ICL mRNA accumulation, then add actinomycin-D to block transcription. The steady-state level of these mRNAs was then examined in the following incubation period and the effect of added sugar examined.

For this experiment, roots from seedlings 5 days after germination were excised and starved for 4 days to accumulate mRNA level. These starved roots were then divided equally and actinomycin-D was added. The roots were then incubated in the absence and presence of 25 mM sucrose for 3, 6, 12 and 24 h. After such intervals, the roots were collected and total RNA was isolated and subjected to northern blot hybridisation in which the filter was hybridised with the *Ms* cDNA probe. A replicate RNA gel was hybridised with the *Icl* cDNA probe. The results are displayed in Figure 5.4. The bands indicated in lane 1 are the MS and ICL mRNAs from RNA of 3 day cotyledons which served as a marker. After 4 days starvation, the level of both mRNAs accumulated to a significant amount (lane 2). Both mRNA levels clearly decrease in amount by approximately 80%

Figure 5.4 Effect of sucrose on MS and ICL mRNA stability. Roots were excised and starved for 4 days (O), followed by incubation with actinomycin-D in the presence and absence of sucrose. RNA was isolated and subjected to northern hybridisation. Lane 1: RNA from cotyledons (C) of germinating seeds. 10 $\mu$ g of total RNA was loaded per track.





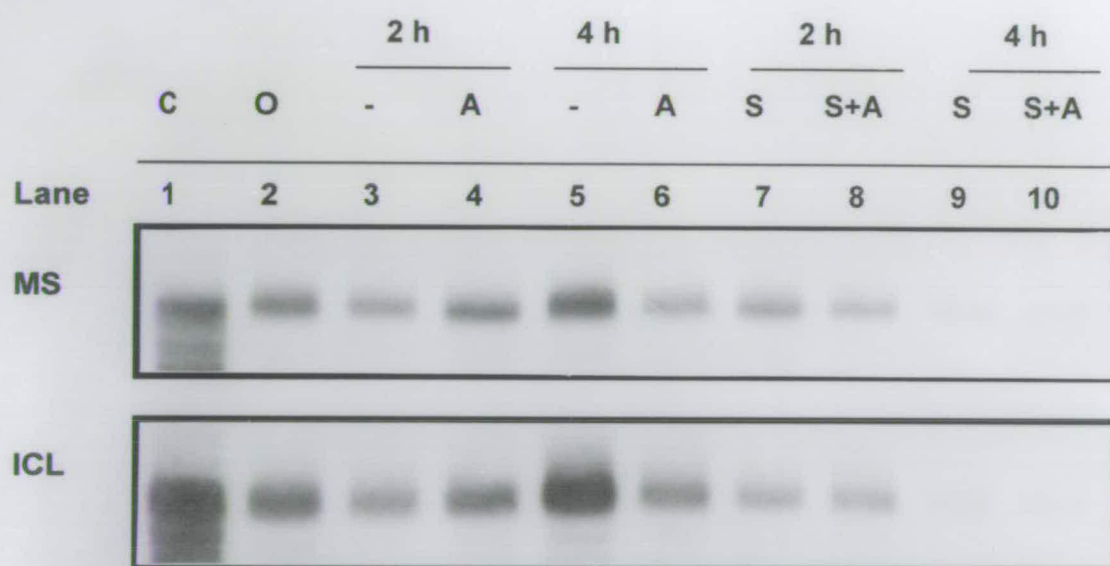
within 3 h blockage of transcription by actinomycin-D treatment (lane 3). The amount of these mRNAs do not apparently decrease further after 6, 12 and 24 h (lanes 4-6). However in the presence of sucrose, the level of mRNA was dramatically reduced within 3 hours (lane 7) and remains essentially undetectable up to 24 h (lanes 8-10).

In order to extend the analysis of mRNA stability, a second experiment was set up to examine changes after 2 and 4 h and to include controls lacking from the first experiment. The roots from 5 day seedlings were excised and starved for 4 days for accumulation of MS and ICL mRNAs. These roots were then incubated for 2 and 4 h in the presence and absence of sucrose and in the presence and absence of actinomycin-D. Roots were collected and total RNA was isolated and subjected to northern blot hybridisation. Replicate RNA was hybridised with the *Icl* cDNA probe. The results are displayed in Figure 5.5. Lane 1 shows MS and ICL mRNA bands from 3 day cotyledon RNA which served as a marker. After 4 days starvation, the mRNA accumulated to a significant amount (lane 2). The level of mRNA changed very little after 2 h whether or not sucrose or actinomycin-D are present (lanes 3,4,7 and 8). However, after 4 h, actinomycin-D leads to a small loss in mRNA in starved roots (lane 6), while addition of sucrose causes a very clear loss in mRNA (lane 9).

### **5.5. The effect of actinomycin-D on inhibiting the *Ms* and *Icl* gene transcription**

All the above experiments assume that actinomycin-D is functioning to inhibit transcription, but this was not directly demonstrated in these experiments. Therefore, further experiments were required to demonstrate that actinomycin-D blocks expression of genes under the conditions employed above and during the

Figure 5.5 Time course of MS and ICL mRNA stability. Roots were excised and starved for 4 days (O), followed by incubation for 2 and 4 h in the presence (S) and absence (-) of sucrose and in the presence (A) and absence (-) of actinomycin-D. RNA was isolated and subjected to northern hybridisation. Lane 1: RNA from cotyledons (C) of germinating seeds. 10  $\mu$ g of total RNA was loaded per track.





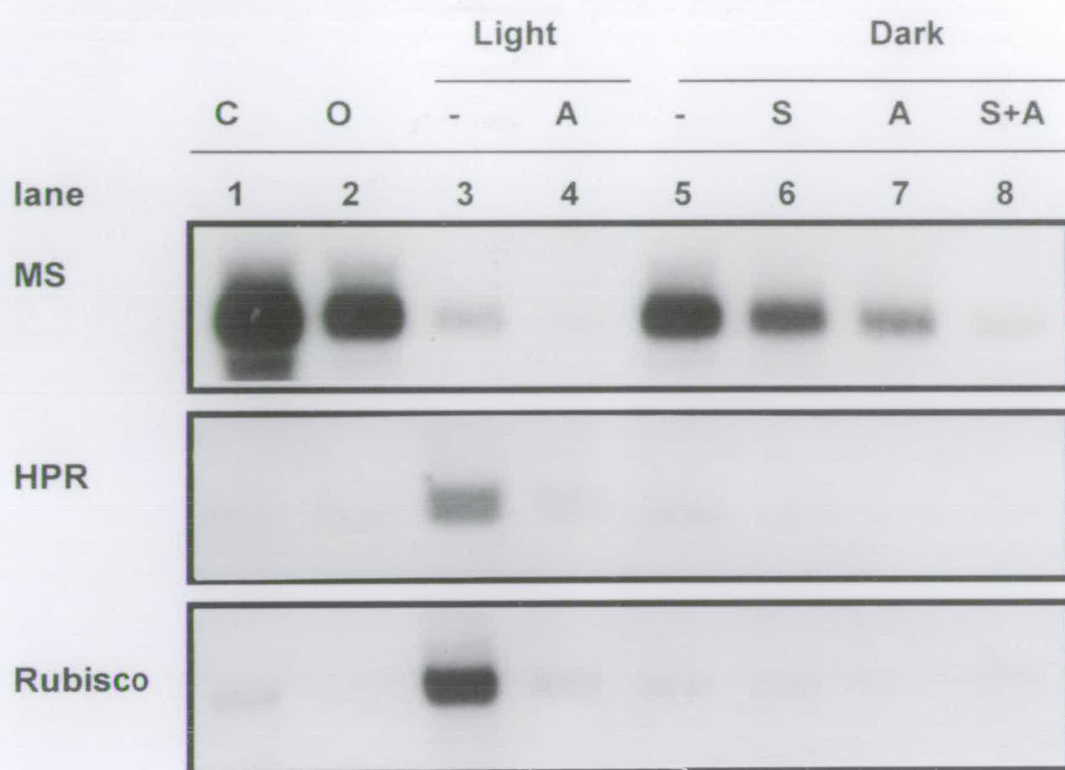
time scale studied. On the basis of the above studies, an experiment was carried out to demonstrate the effectiveness of actinomycin-D to inhibit transcription. A calmodulin cDNA probe was employed in northern blots but mRNA levels were not affected by starvation or sucrose, so no effect of actinomycin-D could be examined. It was then reasoned that the best way to demonstrate the effectiveness of actinomycin-D was to show that it blocked expression of genes whose transcription is activated by sugar treatments of roots. Thus, heterologous nitrate reductase and invertase cDNA probes were employed in further experiments but neither probe could detect mRNA production in response to added sucrose. This may be because these genes are not expressed in cucumber roots or because the heterologous nitrate reductase cDNA from *Cucurbita maxima* and invertase cDNA from *Arabidopsis* could not detect the mRNA under the condition employed.

As an alternative approach, expanded cucumber cotyledons were removed from green seedlings 14 days after germination and incubated in the dark for 4 days to induce starvation and *Ms* and *Icl* gene expression. After starvation treatment, cotyledons were incubated for a further 4 h in the dark in the absence and presence of 25 mM sucrose and in the absence and presence of actinomycin-D. For light treatment, the starved cotyledons were also incubated for 4 h in water with and without actinomycin-D. Total RNA was isolated and northern blot hybridisation was carried out using the *Ms* cDNA probe. Replicate gels were hybridised with a full length hydroxypyruvate reductase (*HPR*) cDNA probe (Greenler *et al.*, 1989) and *Rubisco* cDNA probe (Greenland *et al.*, 1987).

The results are shown in Figure 5.6. For dark incubation treatment (lanes 5-8), the level of *MS* mRNA reduced after 4 h incubation in the presence of actinomycin-D (compare lane 7 with lane 2), suggesting although not conclusively, inhibition mechanism of mRNA transcription by actinomycin-D.

Figure 5.6 Effect of actinomycin-D on MS, HPR and Rubisco mRNA accumulation. Cotyledons were excised and incubated in the dark for 4 days (O). Some cotyledons were incubated in the light in the presence (A) and absence (-) of actinomycin-D, whereas other cotyledons were incubated in the dark in the presence (S) and absence (-) of sucrose and in the presence (A) and absence (-) of actinomycin-D. Incubation period was 4 h. RNA was isolated and subjected to northern hybridisation. Lane 1: RNA from cotyledons (C) of germinating seeds. 10µg of total RNA was loaded per track.





Furthermore, in the presense of sucrose the level of MS mRNA was almost abolished (lane 8). In contrast, when the cotyledons were incubated in the light for 4 h, the level of MS mRNA declined drastically (lane 3) and in the presence of actinomycin-D almost no MS mRNA was detected (lane 4). These data show that the results for *Ms* and *Icl* expression in roots and cotyledons are very similar.

In order to confirm that actinomycin-D had effectively stopped transcription, the filter was hybridised with light-induced HPR and Rubisco cDNA probes. The results presented here demonstrated that the level of HPR and Rubisco mRNAs were very low in 4 days starved cotyledons, but subsequently increase significantly within 4 h in the cotyledons incubated in the light (lane 3). However, in the presence of actinomycin-D accumulation of mRNA was effectively inhibited. These results provide evidence that actinomycin-D does block gene transcription at the concentration and under the conditions employed.

## 5.6. DISCUSSION

Gene expression is a complex multistep process fundamental to all phases of plant growth and development. The first step in this process, transcription of DNA to RNA has so far been the main focus to analyse gene expression and therefore, is the best understood at the molecular level. Nevertheless, it has become apparent that the transcription process alone is not sufficient to explain the regulation of many plant genes. This is because following transcription, the RNA is processed in the nucleus, exported to cytoplasm, bound to ribosomes and translated to a particular protein before being degraded by cellular ribonuclease. Each of these events provides a potential target for posttranscriptional control.



### 5.6.1. Effect of protein phosphorylation and the translation process on derepression of *Ms* and *Icl* gene expression

The regulation of *Ms* and *Icl* gene expression at the transcriptional level has so far been well documented but how it is controlled at the posttranscriptional level was not investigated. Therefore, the experiments described in this chapter were aimed at investigating potential mechanisms other than the transcriptional process which may regulate *Ms* and *Icl* gene expression. The investigation began with the studies on possible effects of protein phosphorylation and translation process in regulating gene expression. However, it then lead to a consideration of seeking a possible role of sucrose in the posttranscriptional regulation of *Ms* and *Icl* genes by affecting their mRNA stability.

One mechanism of signal transduction pathway that has been known to affect gene expression is protein phosphorylation. Previous experiments using the rice  $\alpha$ -amylase gene, whose expression is regulated by sugar, showed that okadaic acid, a potent and specific inhibitor for protein serine/threonine phosphatases, induced  $\alpha$ -amylase expression (Lue *et al.*, 1994). Therefore an experiment was carried out to investigate whether a similar signal transduction mechanism plays a role in *Ms* and *Icl* expression. Results shows in Figure 5.1 indicated no apparent affect of okadaic acid on the expression of *Icl* gene. Therefore, no evidence for involvement of phosphoprotein phosphatases PP1 and PP2A could be observed, which is in contrast to  $\alpha$ -amylase.

In view of this negative result with okadaic acid, rather than proceed to investigate other inhibitors of protein kinases or phosphatases such as calyculin A, it was then decided to determine if other aspects of  $\alpha$ -amylase gene control might apply also to *Ms* and *Icl* genes. The next step was to investigate the relation between mRNA stability and translation using cycloheximide. In eukaryotes,

cycloheximide stabilises most unstable transcripts, either through blocking translation of the transcript itself, or by preventing translation of a labile protein required for mRNA degradation. In the  $\alpha$ -amylase gene, cycloheximide was reported to enhance  $\alpha$ -amylase mRNA accumulation either in the presence or absence of sucrose (Sheu *et al.*, 1994). Nevertheless, as shown in Figure 5.2, *Ms* and *Icl* expression seems not to be affected by cycloheximide. Although this experiment lacks some controls, it does indicate the *Ms* and *Icl* genes do not respond to cycloheximide in the same way as the  $\alpha$ -amylase gene in rice. Further experiments would require controls carried out in the absence of cycloheximide and studies of the expression of *Ms* and *Icl* and  $\alpha$ -amylase genes in the same plant species (cucumber or rice).

#### **5.6.2. Effect of MS and ICL mRNAs stability on gene expression**

However, since no effect of cycloheximide was apparent in cucumber, subsequent experiments were aimed at investigating MS and ICL mRNAs stability in cucumber roots in the absence and presence of sugar. The *Ms* and *Icl* genes are similar to  $\alpha$ -amylase in the way that sucrose transcriptionally down regulates their expression. At the posttranscriptional level, glucose affects the  $\alpha$ -amylase mRNA stability by rapidly reducing its half-life from 12 h in the cells starved of sucrose to less than 1 hour in cells provided with sucrose (Sheu *et al.*, 1994).

The result in Figure 5.4 shows a possible role of sucrose affecting MS and ICL mRNA stability. In the presence of actinomycin-D, the level of mRNA reduced by about 50% within 3 h. This decrease in mRNA level after 3 h could be explained by the blocking of transcription by actinomycin-D and subsequent turnover of mRNA. The level of mRNA was maintained thereafter suggesting that actinomycin-D is only partially effective and mRNA breakdown is matched by a

low level of transcription. Another possible explanation is that further mRNA breakdown after 3 h might require continual gene expression which is blocked by actinomycin-D. When sucrose was added, a rapid loss of mRNA was observed within 3 h incubation and remains undetectable thereafter, confirming that of Figure 5.3. Assuming that actinomycin-D is effectively blocking transcription, this result indicates that MS and ICL mRNAs stability is greatly reduced by sucrose (Figure 5.4, compare lane 3 and 7). While it is apparent that half-life of MS and ICL mRNAs is much less in the presence than in the absence of sucrose, it is not possible to estimate the half-lives from this experiment. The half-life in the absence of sucrose is apparently in the order of 3 h or less, while in the presence of sucrose it is much less. A further experiment was carried out and the result shown in Figure 5.5 strengthen the previous experiment (Figure 5.4) by demonstrating that the effect of sucrose on mRNA stability contributes to the control of *Ms* and *Icl* gene expression (compare lane 6 and 10). Sucrose together with actinomycin-D does not cause a greater loss in mRNA than does sucrose alone (lane 7-10). This can be explained in terms of sucrose acting to inhibit transcription of *Ms* and *Icl* genes and to destabilise mRNAs. Collectively, the result in Figure 5.3, 5.4 and 5.5 suggest that mRNA half-life in the absence of sucrose is significantly greater than in the presence of sucrose. In this respect, there are close parallel with the  $\alpha$ -amylase gene of rice. However, it has not been possible to estimate half-life. In Figure 5.5, actinomycin-D and sucrose had little effect after 2 h. It may be that a certain time is required for sucrose and actinomycin-D to be taken up by the roots before they begin to exert their effects.

Result in Figure 5.6 show that the MS mRNA level was reduced in response to the presence of actinomycin-D (compare lane 5 and 7), but this does not conclusively prove the blockage of transcription. Nevertheless, by using the light-induced *HPR* and *Rubisco* cDNA probes, the expression of both genes was induced within 4 h incubation in the light but was dramatically inhibited in the

presence of actinomycin-D (Figure 5.6a and b, compare lane 3 and 4). This result provides strong evidence of the effectiveness of actinomycin-D to inhibit transcription, and supports the conclusion that MS and ICL mRNAs are destabilised by sucrose.



## **CHAPTER SIX**

### **GENERAL DISCUSSION**

## CHAPTER SIX

### GENERAL DISCUSSION

#### 6.1. *Ms* and *Icl* expression in roots system

The objective of the work presented in Chapter 3 was to investigate the expression of *Ms* and *Icl* genes in cucumber roots. Northern analysis showed that these genes were induced in the absence of sugar and were repressed when sugar was added, showing a similar response to that expressed in other organs such as cotyledons (McLaughlin and Smith, 1994; Kim and Smith, 1994a/b) and cultured cells of cucumber (Graham *et al.*, 1994a). The expression of *Ms* and *Icl* genes were then analysed using hairy roots of cucumber which were successfully obtained by *Agrobacterium rhizogenes*-mediated transformation. A similar pattern of expression was observed as in wild type roots. The availability of hairy roots raised the possibility of studying the expression of *Ms* and *Icl* genes in a homologous system since the whole transgenic cucumber plant is difficult to produce, grow and analyse.

Repression of MS and ICL mRNA accumulation by sucrose suggested that transcriptional control was involved in the metabolic regulation of *Ms* and *Icl* genes expression. It was demonstrated that the 1076 bp *Ms* and 2900 bp *Icl* promoters directed the expression of the GUS reporter gene in transgenic hairy roots. Furthermore, the expression of these constructs was inhibited in response to added sugar, suggesting that regulation of *Ms* and *Icl* expression by carbohydrate involves a transcriptional control mechanism. However, in both cases, the chimeric genes will produce transcripts containing the 5' untranslated region (UTR) of MS and ICL mRNAs, which could potentially be involved in regulation of mRNA

level. Histochemical analysis revealed particular tissues or cells in which the *Ms* and *Icl* promoter directed the expression of the GUS gene. In addition to that, the experiment was also carried out to investigate *Ms* and *Icl* gene expression in roots of whole plants, since this has not been investigated before. Both shading the plant and leaf defoliation were expected to reduce the carbohydrate supply from the shoots to roots. As a result of this treatment, *Ms* and *Icl* gene expression in roots was activated and was accompanied by a reduction in root sugar content, suggesting a correlation between the level of expression and endogenous sugar content. This result revealed the similarity in *Icl* and *Ms* gene expression under carbohydrate deprivation in detached and intact roots.

## **6.2. The function of the glyoxylate cycle during carbohydrate deprivation**

Tissues subjected to carbohydrate deprivation undergo a series of changes in metabolism. Carbohydrate reserves are first utilised, then lipid and protein are used as respiratory substrates, in a process of controlled autophagy (Brouquisse *et al.*, 1991; Dieuaide *et al.*, 1992; Journet *et al.*, 1986). The respiratory rate declines, but a low level of glucose may be maintained, and the changes are reversible by adding glucose (Brouquisse *et al.*, 1991; Journet *et al.*, 1986). The first phase of the response is one of adaptation or acclimation to the change in respiratory substrate. During this time, nitrogen is conserved mainly as asparagine (Brouquisse *et al.*, 1992; Genix *et al.*, 1990).

Subsequently, respiration rate and glucose levels decline further, and asparagine becomes utilised. This is the survival phase, and is followed by the cell disorganisation and death phase which is irreversible (Brouquisse *et al.*, 1992).

Studies with cucumber callus cultures deprived of sugar show that ICL and MS are synthesised early in the response, as the respiration rate and intracellular sugar content are still declining (Graham *et al.*, 1994a). ICL and MS accumulate in detached cucumber cotyledons before sucrose, protein and lipid decline significantly in amount (McLaughlin and Smith, 1994). MS appears in glucose-deprived maize root tips during the acclimation phase (Dieuaide *et al.*, 1992). Therefore, the glyoxylate cycle may play a role in adaptation to the change in respiratory substrate from carbohydrate to lipid and protein.

Figure 6.1 summarises the proposed role of the glyoxylate cycle in creating 4-carbon acids from the breakdown of lipids and proteins in carbohydrate-deprived cells. Acetyl CoA can be generated by peroxisomal  $\beta$ -oxidation of fatty acids (Dieuaide *et al.*, 1992) and amino acid carbon skeletons (Gerbling and Gerhardt, 1988). Furthermore, glyoxylate can be introduced into the cycle from glycine. The cycle results in the net production of one 4-carbon molecule from two molecules of acetyl CoA. Succinate, malate and oxaloacetate (OAA) are interconverted by cytosolic and mitochondrial enzymes (Figure 6.1), but the fate of OAA is considered crucial to understanding the role of the glyoxylate cycle in carbohydrate-deprived cells. Potentially OAA can serve as (1) substrate for PEP carboxykinase in the gluconeogenic pathway, (2) carbon skeleton for asparagine accumulation, and (3) substrate for complete oxidation in the mitochondrion (Figure 6.1). The glyoxylate cycle therefore potentially has gluconeogenic, anaplerotic and respiratory functions.

It has been argued that in such carbohydrate-deprived tissues, gluconeogenesis does not take place to a significant extent because cells maintained on malate or succinate remain depleted in sugar content (Graham *et al.*, 1994a), and the PEP carboxykinase gene is not activated coordinately with *Icl* and *Ms* genes in detached cucumber cotyledons (Kim and Smith, 1994a). However,

**Figure 6.1** Potential roles of glyoxylate cycle during carbohydrate deprivation in plants.

The path of carbon in the glyoxylate cycle is based on that proposed by Mettler and Beevers ( 1980 ). For simplicity, the compensating shuttle of 2- oxoglutarate and glutamate to maintain nitrogen balance between peroxisome and mitochondrion, and the fate of nitrogen and reducing equivalents, are not shown. The correct stoichiometry of reactants is not shown because this will depend upon the extent to which different pathways operate. The figure recognises that aconitase is not localised in the peroxisome ( Courtois-Verniquet and Douce, 1993; De Bellis *et.al.*, 1994 ) and shows the involvement of the cytosol in the conversion of citrate to isocitrate. Closed arrows show reactions, while open arrows show transport of metabolites. Reactions enclosed within the broken line are those involved in complete respiration of 4-carbon acids from the glyoxylate cycle. Operation of the TCA cycle assumes that acetyl CoA is derived from alanine or from malate by the actions of  $\text{NAD}^+$ -malic enzyme and pyruvate dehydrogenase, but other sources are possible ( see text ). The broken line from phosphoenolpyruvate (PEP) to sugars indicates a multi-step process. Control of the fate of 4-carbon acids from the glyoxylate cycle may depend on the extent to which oxaloacetate serves as substrate for (1) gluconeogenesis, (2) asparagine synthesis, and (3) respiration.





lack of sugar accumulation (Graham *et al.*, 1994a) does not necessarily indicate lack of gluconeogenesis and PEP carboxykinase activity was not measured (Kim and Smith, 1994a). Wanner *et al.* (1991) showed that a small amount of gluconeogenesis from radiolabelled galactolipid may be possible in detached barley leaves. Stewart and Beevers (1967) showed that gluconeogenesis from amino acids occurs in castor bean endosperm. The possibility that the glyoxylate cycle serves a gluconeogenic role in carbohydrate-deprived cells therefore requires further examination. Such cells are still engaged in biosynthesis, which may require a supply of sugar phosphates.

It has been argued that the glyoxylate cycle may serve an anaplerotic role in carbohydrate-deprived cells, to replenish 4-carbon acids in the mitochondrial citric acid cycle (Graham *et al.*, 1994a; Kim and Smith, 1994a). Figure 6.1 shows how the glyoxylate cycle could function in asparagine accumulation. The possible respiratory role of the glyoxylate cycle is also illustrated in Figure 6.1. OAA could be completely respired by the mitochondrial citric acid cycle, provided that a supply of acetyl CoA is maintained for citrate synthase activity. The acetyl CoA could potentially be produced from several different sources including the combined actions of NAD<sup>+</sup> malic enzyme and pyruvate dehydrogenase, or from alanine (Figure 6.1), from glycerol entering the glycolytic pathway, and from mitochondrial  $\beta$ -oxidation which is known to occur in carbohydrate-deprived cells (Dieuaide *et al.*, 1993).

Potentially, the glyoxylate cycle could function in gluconeogenic, anaplerotic and respiratory roles, but to different extents, which may change as the cells pass through acclimation and survival phases to death. The possibility of metabolite channeling of glyoxylate cycle products into particular products should also be considered. The function of the cycle could be revealed by following the fate of radiolabelled substrates which are specific to the cycle (glyoxylate in

particular), and through the isolation of mutants which lack a functional glyoxylate cycle in carbohydrate-deprived cells. Whatever the function, it is possible that the cycle has an important role to play in adaptation to carbohydrate deprivation, that this provides an adaptive advantage to plants, and may occur commonly in nature.

### **6.3 Using hairy roots to study *Ms* and *Icl* gene expression**

In Chapter 3, the experiments that were carried out have clearly shown that *Ms* and *Icl* genes are expressed in cucumber roots. Interestingly, the analysis could then be carried out in hairy roots of cucumber. The *Ms* and *Icl* genes in hairy roots were found to be as responsive as normal roots to sugar regulation. These results led to the consideration of studying the expression of *Ms* and *Icl* genes in a homologous transgenic expression system. Even though the *cis*-acting elements of the *Ms* gene required for germination and sugar response had been putatively identified, these were then analysed in transgenic hairy roots to investigate their effectiveness as a homologous host system. The result showed that the region between -233 bp and -216 bp upstream of transcription start is required for the sugar response, which agrees well with the previous experiment using heterologous transgenic *Nicotiana* seedlings (Sarah *et al.*, 1996). The value of hairy roots as a system to study gene expression was also shown by analysis of a 191 bp AluI-AluI fragment, which spans from the position of -248 to -125 bp relative to the start of transcription, previously shown to direct expression of the *Ms* construct under metabolic regulation.

On the basis of these data, hairy roots were then used to identify *cis*-acting elements responsible for *Icl* gene expression. It was demonstrated that there are at least two different *cis*-acting elements necessary for sugar and germination

responses. The region between -1568 and -1367 bp upstream of translation start was required for the sugar response whereas sequences upstream of -1568 bp were required for the germination response. An important observation was that the region -572 bp upstream of the translation start was capable of directing a low level of germination response. This was shown by the spatial and temporal expression in cotyledons. In conclusion, at least two different *cis*-acting elements were shown to be required for the germination and sugar responses in the cucumber *Icl* gene, and different interacting *cis*-sequences were implicated in the germination response. These separate quantitative and qualitative germination response sequences were not observed in the *Ms* gene promoter (Sarah *et al.*, 1996).

Comparison between *Ms* and *Icl* genes of cucumber has revealed a number of sequences which show similarity, and are called IMH sequences (Reynold and Smith, 1995b). It was suggested that IMH2 could have a role in the sugar response. Nevertheless, the region between -1568 and -1367 bp necessary for the sugar response of the *Icl* gene does not contain IMH2 (figure 4.9a and b). One possible explanation is that IMH2 might require sequences upstream to confer the sugar response. So far, analysis of *Ms* and *Icl* gene expression has been largely confined to deletion analysis (with the exception of the *Ms* Alu1-Alu1 fragment). The other approach of analysis would be gain of function analysis as discussed in Chapter 4.

To this end, potential germination and sugar response regions in *Icl* and *Ms* promoters of cucumber have been identified. The future task will be to identify more precisely the sequences of the promoter regions by site-directed mutagenesis and to isolate mutants in which *Ms* and *Icl* are not expressed during starvation. This may help to determine DNA sequences needed for interaction with specific proteins which may involved in regulating *Icl* and *Ms* gene expression. Once the sequences have been identified, this will then lead to approaches to isolate

transcription factors which recognise such sequences in the promoter regions or in the other gene-flanking region. For this purpose, *in vitro* 'footprinting' employing two nucleases, exonuclease III and DNase I is used to identify the site where proteins are bound to DNA and gel retardation assay to identify DNA binding proteins. These methods will allow the sites of protein-DNA complexes to be mapped and as well as allowing identification of protein binding regions in DNA, may permit purification of the protein that is bound to the DNA. One factor has so far been shown to bind to IMH1 (Graham *et al.*, 1994b). Techniques such as these can be useful to study interaction of transcription factors with the DNA and with other proteins, thus further elucidating the mechanism controlling *Icl* and *Ms* gene expression. Ultimately, it may be possible to identify signal transduction components which regulate the activity of such proteins. Expression of *Ms* and *Icl* genes in *S. cerevisiae* (Witt *et al.*, 1966) is also regulated by carbon catabolite repression, suggesting that there might be a common control mechanism. Comparison of the *Icl* and *Ms* promoter regions necessary for expression with those of *S. cerevisiae* should provide further information regarding conserved regulatory regions.

#### **6.4. *Ms* and *Icl* expression at the posttranscriptional level**

In Chapter 3, expression of the endogenous *Ms* and *Icl* genes in roots was shown to be coordinately regulated by sucrose apparently at the transcriptional level. This evidence was supported by the expression of the GUS reporter gene directed by the *Ms* and *Icl* promoters. In both cases, these genes were induced in the medium without sugar but were repressed when sucrose was added. Beside the evidence that sucrose affected gene transcription, its possible role in the posttranscriptional regulation of *Ms* and *Icl* genes could not be excluded and thus deserved further investigation. So investigation was focused on the effect of

sucrose on mRNA stability. By using actinomycin-D, a transcriptional inhibitor, evidence was provided to suggest that sucrose reduced the mRNA half-life by destabilising the MS and ICL mRNAs.

A mechanism of carbon catabolite repression by controlling mRNA turnover has been shown in yeast. It was demonstrated that a significant part of the regulation by glucose of the gene encoding the iron-protein subunit (Ip) of succinate dehydrogenase, involved the regulation of the turnover rate of its mRNA. Transcription of the *Ip* gene was repressed by glucose and the half-life of Ip mRNA was greatly reduced in the presence of glucose (Lombardo *et al.*, 1992). Even though the half-lives of MS and ICL mRNAs were not determined in this work, it raised an exciting prospect of studying a system in which half-lives of these mRNAs could easily be controlled by manipulation of the media. Another aspect that needs attention in order to further investigate stability of MS and ICL mRNAs stability is the transcription rate, which was not investigated in this work. For the  $\alpha$ -amylase gene, a discrepancy between nuclear run-on transcription measured *in vitro* and mRNA accumulation *in vivo* generally showed that the transcript is most likely to be controlled at the level of mRNA stability (Sheu *et al.*, 1994). The prevalence of such discrepancies and observations documenting the effect of transcribed sequences on *fed-1* and SAUR mRNA accumulation (Dicky *et al.*, 1992; Newman *et al.*, 1993) indicate that the control of mRNA stability could be wide spread in plants.

Another approach to study mRNA stability that could be worth pursuing is to use promoters subject to transient induction or repression. The advantage of this approach is that it does not disrupt normal cell growth and has little or no effect on other cellular processes. In *E.coli*, the *trp* promoter which can be repressed by adding tryptophan has been used for this purpose (Morse *et al.*, 1969). Similarly, in yeast, transcription from the *GAL1* promoter can be inhibited by adding glucose

to the medium (Shyu *et al.*, 1989). Basically, the method would need a strong and repressible promoter that can be inserted in front of the *Ms* and *Icl* genes. After a time period of induction, transcription of these transgenes from a particular promoter abruptly ceases, thereby allowing the degradation of the mRNAs to be monitored over time, in the absence of transgene mRNA synthesis. In this manner, the promoter should not be active, thus only looking at the mRNA stability. In plants, one such promoter that has been used was top 10 promoter. This promoter was made by combination of regulatory control elements from different organisms. It contained TetVP16, a fusion between bacterial tetracycline repressor and activation domain of the *Herpes simplex* transcription factor, VP16. In the presence of tetracycline, the TetVP16 is inactivated, resulting in a cessation of transcription from the Top 10 promoter (Weinmann *et al.*, 1994). Another potential promoter that could be used is 35S CaMV. In this system, while the 35S promoter is not being affected, the MS and ICL mRNA steady state level can be examined in the presence and absence of sugar.

*Ms* and *Icl* genes are also regulated at the level of translation and posttranslational. Studies on MS and ICL in embryos and seedlings of *B. napus* showed that the relative accumulation of the proteins and enzyme activities do not correspond to the mRNA levels. The ratio of MS protein to mRNA is much higher than that of ICL but the *in-vitro* translation experiments showed that these differences do not appear to account for the discrepancy between mRNA and protein levels (Ettinger and Harada, 1990). In cucumber plant, the temporal and spatial pattern of ICL and MS mRNA accumulation suggest that the two genes are coordinately regulated, but whether their accumulation occurs at the same level and reflects proportional differences in enzyme accumulation remains to be elucidated. In order to resolve this issue, work should also concentrate on the quantification of MS and ICL enzyme activities and protein following mRNA



translation. This will likely tell whether translational and posttranslational processes may affect MS and ICL accumulation.

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## **Publications \***

Two papers have been accepted and are in press as a result of part of this work. These will appear in **Plant Molecular Biology and Gene**.

1. Ismail I., De Bellis L., Alpi A. and Smith S. M. (1997) Expression of glyoxylate cycle genes in cucumber roots responds to sugar supply and can be activated by shading or defoliation of shoot. *Plant Mol. Biol.* In press.
2. De Bellis L., Ismail I., Reynolds S. J., Barrett M. D. and Smith S. M. (1997) Distinct *cis*-acting sequences are required for the germination and sugar responses of the cucumber isocitrate lyase gene. *Gene*, In press.